Transplant Arteriosclerosis and In-Stent Restenosis

Onuta, Geanina

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Experimental studies on pathomechanisms and therapeutic intervention

Geanina Onuta

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For my family

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Abstract

Vascular remodeling is a broadly used term which describes structural changes of the arterial wall as a response to various stimuli resulting in the narrowing of the vessel lumen and subsequent downstream tissue ischemia. Transplant Arteriosclerosis (TA) and in-stent restenosis (ISR) are two major vascular diseases characterized by obliterative vascular remodeling and may be regarded as healing processes within the vascular wall that, however, proceed beyond the needs of functional repair leading to a vascular maladaptive status. The common hallmark of these obliterative vascular diseases is intimal thickening resulting from both an increase in the number of cells in the subendothelial intima (*i.e.* vascular smooth muscle cell proliferation) as well as the amount of extracellular matrix proteins, culminating in neointima (NI) formation. Various insults to the vascular bed result in endothelial injury which constitutes the initiating stimuli resulting in luminal narrowing and impaired vascular function. As yet, no adequate preventive or curative therapies are available to prevent NI formation in TA and ISR. This *General Introduction* focuses on the cellular and molecular mechanisms involved in NI formation in TA and ISR. Although these diseases present many similarities, they represent distinct forms of vascular remodeling processes. Similarities and differences between the underlying pathogenetic mechanisms are being discussed and potential treatment strategies are being presented. Particular emphasis is given to endothelial and smooth muscle vascular progenitor cells and their role in vessel homeostasis as well as their potential as novel therapeutic targets.

Abbreviations

EC endothelial cell
EPC endothelial progenitor cell
ECM extracellular matrix
ISR in-stent restenosis
NI neointima
SMPC smooth muscle progenitor cell
TA transplant arteriosclerosis
VSMC vascular smooth muscle cell

Vascular remodeling and neointima formation

Vascular remodeling is a broadly used term which describes structural changes of the arterial wall as a response to various stimuli, such as wall shear stress, hypoxia, and immunological or mechanical injuries, that lead to changes in vessel size and luminal width.[1] The complex set of vascular changes includes altered phenotype and localization as well as function of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). This coincides with changes in extracellular matrix (ECM) composition.[2] Vascular remodeling is an active process of structural modification in which at least four cellular processes are involved: cell proliferation, cell death, cell migration, and production or degradation of ECM. Vascular remodeling is dependent on a dynamic interaction between locally generated growth factors, vasoactive substances, and hemodynamic stimuli.[3] Narrowing, thickening and stiffening (*i.e.* loss of elasticity) of the vessel wall will result in reduced blood flow and subsequent ischemia in downstream tissue/organs. Vascular remodeling has various clinical and pathological manifestations, including spontaneous (native) atherosclerosis, transplant arteriosclerosis (TA), arterial bypass graft atherosclerosis, vein graft stenosis, restenosis after percutaneous transluminal coronary angioplasty, and in-stent restenosis (ISR). The common hallmark of these obliterative vascular pathologies is intimal thickening resulting from both an increase in the number of cells in the subendothelial intima as well as the amount of ECM.[4] As yet, no adequate preventive or curative therapies are available to treat NI formation in TA and ISR. The NI consists of VSMCs, ECM and inflammatory cells[5] forming a fibrotic layer covered at the luminal side by endothelium. In all these inflammatory, proliferative, and migratory events a primary (common) pathophysiologic mechanism supposedly underlies uncontrolled VSMC proliferation as observed in TA and ISR.[6]

Both TA and ISR develop as a result of the interventions performed, in part, to treat occlusive atherosclerotic diseases and end-stage organ dysfunction due to ischemic damage. TA and ISR may be regarded as healing processes within the vascular wall that, however, proceed beyond the needs of functional repair leading to a vascular maladaptive status. We propose that damage of the tissue architecture elicits an evolving defense reparative mechanism aiming for vascular wall re-building. The presence of ongoing perivascular inflammation will however sabotage the initial defense mechanism resulting in excessive pathological repair culminating in vessel obstruction, downstream ischemic tissue damage, and tissue necrosis.

This *General Introduction* focuses on the cellular and molecular mechanisms involved in NI formation in TA and ISR. Similarities and differences between the underlying pathogenetic mechanisms are being discussed and potential targets for therapeutic intervention are being presented. Particular emphasis is given to endothelial and smooth muscle vascular progenitor cells and their role in vessel homeostasis as well as their potential as novel therapeutic targets.

Progenitor cells in vascular remodeling

During vascular remodeling processes both ECs and VSMCs are actively involved. Maintenance of vascular endothelial integrity and repair was historically believed to be regulated exclusively by resident mature vascular cells. However, it is now recognized that populations of vascular progenitor cells in a variety of tissues which are capable of differentiation into ECs and VSMCs, thereby participating in neointima formation and vascular remodeling.[7] Vascular progenitor cells include both endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs). Vascular progenitor cells have been identified in the BM, in the circulation, in the vessel wall, and in various extravascular sites (Figure 1).[8] The BM contains hematopoietic and mesenchymal stem cells which have the ability of self-renewal and differentiation into a variety of cell types including VSMCs.[9-11] Similarly, human peripheral blood monocyte-derived subsets act as pluripotent stem cells and can be induced to acquire macrophage, epithelial, endothelial, neuronal, and hepatocyte phenotypes.[12] Vascular progenitor cells that can differentiate in culture into either endothelial or smooth muscle cells have been isolated from peripheral human blood.[13,14] Besides circulating progenitor cells, vascular wall progenitor cells have been identified in so-called vasculogenic zones in the media[15] as well as in the adventitia[16,17] in rodent and human arteries. The vascular progenitor cells in tunica media of healthy adult mice were Sca-1^{+}, c-kit^{low} and CD34^{low}, and were able to undergo EC and VSMC differentiation when exposed to VEGF and TGB-β1/PDGF-BB, respectively.[15] Similarly, vascular progenitor cells residing in the mouse aorta adventitia express Sca-1 and differentiate *in vitro* into ECs and VSMCs when exposed to VEGF-A and PDGF-BB, respectively.[17] In human vessels a population of CD31^{-}CD34^{+} cells has been identified at the border of the media and adventitia of large- and medium-sized arteries and veins.[16,18,19] Within the vascular wall, the adventitia is not considered anymore as just a layer of connective tissue supporting the blood vessel, but rather as an active participant in vascular progenitor cell transfer by providing progenitor cell niches and contribution to the movement of these cells between BM, circulation and vessel wall.[20] Another location for vascular wall progenitor cells, which has been reported with pericytic properties, is the subendothelial zone.[21] Based on these data, there is evidence for the existence of BM-derived recirculating and resident vascular wall progenitor cells, with a direct and indirect role, due to paracrine mechanisms, in vascular remodeling. It is still unknown whether EPCs and SMPCs are derived from a common ancestral vascular progenitor cell which then may have a dichotomous role in its response to vascular injury: a beneficial role for endothelial repair (EPC) and a detrimental role in neointima formation (SMPC).[22-24] Identification of the exact phenotype of these (recirculating) cells as well as the molecular mechanisms that drive their differentiation is a prerequisite in developing efficacious future strategies aiming at modulating vascular progenitor cell differentiation to attenuate neointima formation.

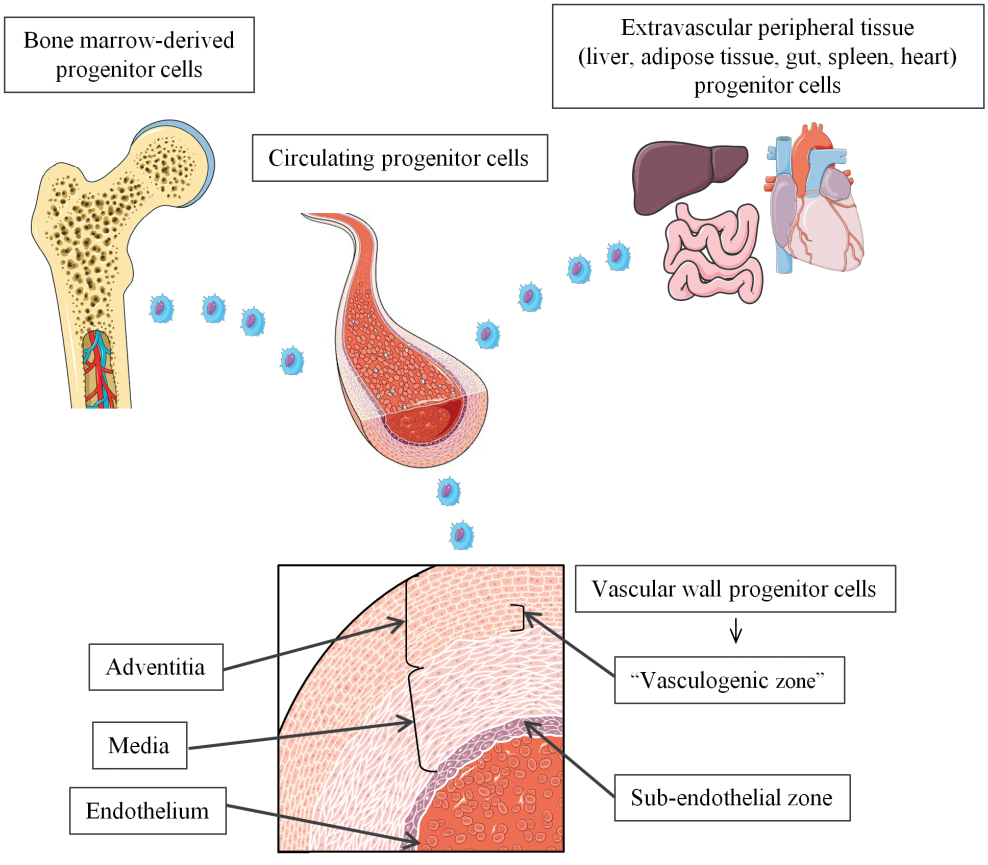

Figure 1. Possible sources of vascular progenitor cells. Vascular progenitor cells have been identified in the bone marrow, in the circulation, in the vessel wall, and in various extravascular sites. There is most likely a continuous movement of cells between the different compartments via the circulation. The lower panel displays a schematic representation of (a part) a cross-section of and artery and shows the different layers of the vessel wall in which the so far identified stem cell niches (such as the "vasculogenic zone" in the adventitia and the sub-endothelial space) are indicated. Figure was produced using Servier Medical Art (www.servier.com).

Transplant arteriosclerosis

Chronic transplant dysfunction and transplant arteriosclerosis

Cardiac transplantation is a widely accepted therapy for the treatment of end-stage congestive heart failure (mostly ischemic and dilated cardiomyopathy). Candidates for cardiac transplantation are patients that are refractory to conventional medical therapy and are excluded from other surgical interventions because of the poor condition of the heart.[25] Current immunosuppressive therapy, of which the mainstay is provided by calcineurin inhibitors (CsA, FK506), is effective to treat acute rejection but not to prevent cardiac allograft vasculopathy which is also referred to as transplant arteriosclerosis (TA). Therapy for TA has thus been disappointing and the only definitive treatment is retransplantation since intragraft revascularization therapy does not result in survival benefit. To date, TA

is the main cause of Chronic Transplant Dysfunction (CTD) and thereby the main factor limiting the long-term success of cardiac transplantation. TA is the second leading cause of death after 1 year following transplantation, second only to malignancy.[26] TA occurs in >50% of heart transplant recipients in the first several years after transplantation.[27] TA is characterized by concentric neointimal proliferation with lumen narrowing and ischemic tissue damage with loss of graft function eventually.

Both alloantigen-dependent and independent factors are described as risk factors for TA development.[28,29] Alloantigen-dependent factors include the number of donor-recipient HLA mismatches and the number, duration and time of onset of rejection episodes.[30,31] Alloantigen-independent risk factors include hyperlipidemia, older donor age, obesity, diabetes mellitus, hypertension, hyperhomocysteinemia, cytomegalovirus infection, ischemia/reperfusion injury, and donor brain death.[28,30-34]

Endothelial and medial smooth muscle cell injury in TA development

The first line of contact between the transplanted donor organ and recipient blood is the graft endothelium. Allogenic graft ECs serve as potent stimuli of the alloimmune response and form a major target of alloreactivity. Various immune-mediated pathways have indeed been implicated in enhanced EC apoptosis.[35] The initial injury to the graft endothelium may start even before transplantation primarily due to donor brain death and ischemia/reperfusion injury. In TA, non-immune and immune-mediated mechanisms initiate apoptosis of ECs, which is then followed by intimal hyperplasia. This sequence of events suggests that damage and subsequent apoptosis of ECs play a central role in NI formation through the activation of at least three, not mutually exclusive, processes.[35] First, endothelial apoptosis induces a thrombogenic and hyperadhesive state for inflammatory cells, thereby facilitating sustained leukocyte transmigration into the subendothelial space resulting in enhanced vascular inflammation which in turn perpetuate the initial EC injury.[36] Secondly, phagocytosis of apoptotic cells by macrophages increases the production of transforming growth factor-β1 (TGF-β1) which is a profibrotic cytokine known to increase NI formation.[37] Thirdly, EC apoptosis triggers ECM proteolysis thereby initiating the production of fibrogenic mediators like TGF-β1, that promote profibrotic responses at the sites of injury.[35,38] Ongoing EC injury in the graft occurs as the recipients' immune response against donor antigens maintains (*i.e.* [subclinical] rejection). Damaged endothelium will expose the underlying medial VSMCs and ECM to the recirculating blood thereby increasing the risk of thrombosis.

It is now well established that the endothelium represents far more than an inert cell layer lining the vessel lumen.[39] Depending on the type of vascular bed, ECs exert various functions including regulation of the vascular tone, coagulation, and inflammatory responses.[40] EC injury manifests at different levels. Depending on the overall endothelial integrity EC injury can be classified as either denuding or non-denuding injury.[41] Denuding injury involves the loss of a substantial area of endothelial coverage. EC denudation is usually noted during significant episodes of acute cellular rejection or vasculitis, as well as during episodes of ischemia of which the latter can occur already during the process of organ preservation and

reperfusion.[42] The non-denuding form of EC injury does not involve a significant loss of endothelial coverage and is characterized by relatively rapid replacement of individual ECs that are injured and lost. Clinically, non-denuding EC injury principally presents as endothelial dysfunction (*i.e.* changes in the concentration of the chemical messengers produced by EC and/or blunting of the nitric oxide (NO)-dependent vasodilatory response).[42,43] Besides EC injury of graft endothelium, organ transplantation also has a generalized, systemic harmful effect on recipients' EC function. Systemic EC dysfunction after transplantation develops not only as a result of the pre-existent clinical condition (such as diabetes), but also develops *de novo* presumedly as a result of the systemic inflammatory burden caused by the ongoing, subclinical rejection response.[44] This transplant-associated systemic EC dysfunction thus occurs outside the transplanted organ and forms a main contributor to the elevated cardiovascular risk that transplant recipients face. Systemic EC dysfunction in transplant recipients is associated with significant morbidity and mortality.[45-47] The majority of renal transplant recipients loose their graft not because of graft failure, but rather due to the development of cardiovascular disease resulting in patient death with a functioning graft.[48]

Programmed VSMC death plays a significant role in remodeling (including TA) of the injured vasculature.[35,49-52] Apoptosis of medial VSMCs is triggered by inflammatory processes.[53-55] The decrease of α-actin expressing medial VSMCs[56-60] impairs the graft vascular wall architecture and vasomotor function. Vascular injury due to chronic inflammation elicits a repair response that involves thrombosis, migration and proliferation of vascular cells, matrix production, and infiltration of inflammatory cells (see below).[3] Cytokine-induced activation and proliferation of VSMCs is considered one of the critical cellular events in TA development and, accordingly, their proliferation is a potential target for therapeutic intervention to attenuate NI formation.[61]

Origin of endothelial and smooth muscle cells in TA

The identification of the origin and phenotype of neointimal cells in TA is pivotal in the development of therapeutic interventions aiming at targeting VSMCs in the development of TA. The response-to-injury hypothesis for the development of atherosclerosis proposes medial origin of intraplaque VSMCs.[62] For a long time, this hypothesis was also applied to the development of TA in allografts. According to this hypothesis, neointimal cells in TA are derived from the medial layer of the vascular wall and end up in the subendothelial space due to migration.[56,63] Arterial VSMCs normally reside in the tunica media in their non-proliferative contractile state. However, upon injury the release of inflammatory mediators results in modulation of the phenotype of donor medial VSMCs, resulting in dedifferentiation from a contractile state to a dedifferentiated synthetic cell with proliferative, migratory and ECM-secreting capacity.[64-67] According to the response-to-injury hypothesis, the VSMCs present in the neointima in TA are thus donor-derived. However, in late 90s of the last century this hypothesis (holding graft origin of neointimal VSMCs) was challenged and there is now, primarily based on data from experimental transplantation in rodents, a well documented evidence for (partly) recipient origin of NI cells in TA.[22,68-74] Consequently, the specific anatomical origin of these recipient-derived NI cells became a matter of

major interest in unraveling the pathogenetic mechanisms underlying TA development. Several animal models have been used to study the origin of NI cells in TA including solid organ transplants (heart, kidney) as well as arterial (aorta) segments. Depending on the experimental transplant model used, different (contradicting) results regarding the origin of NI cells in TA were reported. In our laboratory, Hillebrands *et al.* were able to show that neointimal ECs and VSMCs in a rat aorta transplant model were completely of recipient origin, whereas in a rat heterotopic cardiac transplant model only the neointimal VSMCs turned out to be of recipient origin with preserved graft endothelium.[68,75] Using a rat kidney transplant model we recently demonstrated that both neointimal ECs and VSMCs are of donor origin, whereas ECs in peritubular and glomerular capillaries were of both recipient and donor origin.[76] Based on our observations and data reported by others, we hypothesized that depending on the severity of the initial injury and the donor endothelial cell/resident progenitor cell potential to proliferate/differentiate, endothelial integrity is restored by either donor resources or by recipient-derived (vascular progenitor) cells.[5] The differences in data obtained in different models underscores the importance of both severity of the injury (in the aorta transplant model recipient ECs and medial VSMCs completely disappear with limited remaining capacity for graft-mediated reconstruction) and the organ-specific response to injury (*i.e.* different graft-specific endogenous potential to restore the damaged vessel wall). Indeed, Han *et al.* provided experimental evidence that the bone marrow (BM) compartment can serve as complementary source of neointimal VSMCs during vascular healing using two types of insult in a murine model: endovascular probe scratch injury of the iliac artery and organized arterial thrombus formation into the left common carotid artery.[77] Using BM chimeric mice, the authors demonstrated that BM-derived cells contribute to NI formation only when the media of the iliac artery sustained severe damage, but not in arteries subjected to minimal medial damage.[77]

Various sources of NI VSMCs can thus be identified, including differentiated donor-derived medial VSMCs[15,78], tissue resident donor or recipient-derived vascular progenitor cells[79,80], transdifferentiation of mature donor ECs into VSMCs *(i.e.* endothelial-to-mesenchymal transdifferentiation)[81,82], and influx of recipient-derived recirculating BM and non-BM-derived stem cells.[5,22,69-72,83,84]

In contrast to various experimental studies including our own data[68,75], most studies on the origin of neointimal VSMCs in TA in human cardiac allografts describe that virtually all neointimal VSMCs are donor-derived.[85,86] However, in contrast to human cardiac allografts Grimm *et al.* showed that in human renal allografts the majority of neointimal VSMCs were of recipient-origin.[73] In our laboratory, Boersema *et al.* also recently determined the origin of neointimal ECs and VSMCs in explanted human renal allografts, thereby showing that up to 20% of the neointimal VSMCs were recipient-derived.[74] Whether organ-specific differences might explain the observed differences between the cardiac and renal allografts, remains to be elucidated.

Several phenomena might contribute to the differences observed between the experimental and clinical studies regarding NI cell origin. First, preserved medial VSMCs in the intragraft arteries of transplanted solid allografts may serve as a source for VSMCs

that can repopulate the neointima. Because in rodent vascular transplantation models no or limited (temporarily) immunosuppressive drugs are administered, medial cell destruction often leads to (nearly) complete medial VSMC destruction. In contrast, in human transplantation more aggressive and prolonged immunosuppressive regimen are used to prevent rejection, thereby most likely resulting in protection of complete medial VSMC destruction. One can envision that when viable medial VSMCs remain present, these cells can serve as a source of NI cells, whereas in the case of complete medial destruction, other sources are required by definition. However, in our model of TA in cardiac allografts, a small rim of presumably donor-derived medial VSMCs was present, whereas virtually all neointimal VSMCs were found to be of recipient origin.[68] Another difference between experimental rodent models and the human transplant setting might be the presence of pre-existing vascular disease (*i.e.* native atherosclerosis) in the grafts at the time of engraftment.[87] These intimal lesions contain donor-derived VSMCs that may provide the basis for further outgrowth of VSMCs during the subsequent development of (posttransplant) TA.[85] Such pre-existing intimal lesions are rarely found in animal tissues used for transplantation.

Taken together, experimental and human studies show evidence for both recipient- and donor- derived VSMCs in NI formation. The pathogenetic mechanisms might depend on both the type of transplanted organ as well as the severity and duration of endovascular injury.[5,76]

Atherosclerosis and (in-stent) restenosis

Atherosclerosis

Atherosclerosis is characterized by asymmetric focal thickening of the arterial tunica intima, consisting of VSMCs, inflammatory cells (predominantly T cells and macrophages), ECM components, lipids, and necrotic cellular debris.[88] Atherosclerosis can affect all large and medium-sized arteries and results in luminal narrowing and downstream ischemic tissue damage. Atherosclerosis in the coronary arteries is a major cause of heart failure[89] causing high rates of cardiovascular morbidity and mortality.[90] A major step forward in the treatment of atherosclerosis was the introduction of percutaneous transluminal coronary angioplasty (PTCA) in the late 70s.[91,92] The main complication following PTCA was, however, restenosis development occurring in ~30%-40% of the patients within the first 6 months after intervention.[93] Postangioplasty restenosis results from direct trauma to the artery and is believed to represent an arterial healing response after injury.[94] Postangioplasty restenosis is characterized by vascular elastic recoil, reorganization of thrombi, neointimal proliferation and negative remodeling, *i.e.* reduction in vessel diameter due to inward vessel constriction.[94-97] This negative remodeling has been significantly improved after the introduction of endovascular stenting.

In-stent restenosis (ISR)

Stenting is a method of revascularization of significant obstructive atherosclerosis with already a long history in the treatment of coronary artery disease. However, stenting is also becoming an increasingly used treatment modality in peripheral atherosclerosis in which much higher restenosis rates are observed compared with stenting in coronary arteries.[98-101] Stenting implies endovascular placement of a metal scaffold that eliminates elastic recoil and negative remodeling, thereby preserving the vascular lumen.[102] The accumulating number of reports of reduced restenosis rate after stenting (compared with PTCA)[93,103] led to widespread agreement of coronary stenting as the therapy of first choice for coronary atherosclerosis.[92,104,105] Although results of PTCA have improved substantially over the past decade[106] and stenting significantly reduced angiographic restenosis in comparison with PTCA,[93,103,107] development of ISR still remains an unsolved clinical problem occurring in 15%-30% of stented arteries.[108-111] ISR can be diagnosed both clinically and angiographically. Clinically, ISR after coronary stenting is defined as the presence of recurrent angina or evidence of myocardial ischemia.[112] Angiographically, ISR is considered to be present if lumen diameter loss is > 50%.[94,112]

Risk factors for ISR

Risk factors for ISR are summarized in Table 1. The predictors of ISR are various in nature, and are related to both patient-dependent factors, and lesion- and procedure-dependent factors. In particular diabetes mellitus (DM) is considered as a major risk factor for restenosis after both bare-metal and drug-eluting stenting.[113-120] In a retrospective study by Abizaid *et al.* the restenosis rate was 16.3% for patients without diabetes, 17.6% for patients with Type 2 diabetes, and 28% for patients with Type 1 diabetes.[121] In a meta-analysis of six studies including a total number of 1166 patients with diabetes (predominantly Type 2 diabetes) and 5070 non-diabetics, Gilbert *et al.* reported an average restenosis rate of 36.7% and 25.9% in diabetics and non-diabetics, respectively.[122] In this study, restenosis rates were also shown to correlate with age. After stratification for age, the authors demonstrated that the diabetic patients were older and that the incidence of restenosis after stenting was reduced approximately 50% after adjusting for age which led to the conclusion that the apparent effect of diabetes on restenosis might be overstated.[122] West *et al.* retrospectively analyzed all stented diabetic patients in 16 studies of percutaneous coronary interventions.[123] Within these studies, 418 of 3090 (14%) of the stented patients with 6-month angiographic follow-up had DM. Restenosis occurred in 21% of the non-DM and 31% of the DM patients, with no significant difference in age between the two groups. Loutfi *et al.* suggested that an important contributor to the increased need for repeated revascularization intervention among diabetics is not only restenosis but also progression of coronary atherosclerotic disease.[124] Therefore, it appears reasonable to assume from the numerous studies conducted, that DM indeed predisposes for ISR although the magnitude of this effect of diabetes is still inconclusive.[91]

Individuals with DM usually present with signs of accelerated atherosclerosis (*i.e.* macrovascular disease) manifested by acute coronary syndromes, myocardial infarction

Table 1. Risk factors for the development of in-stent restenosis after stenting

Risk factor	System involved		References
patient-dependent risk factors	metabolic disorders	diabetes mellitus	110,114,121,281
	RAAS system	higher plasma angiotensin-converting enzyme levels	116
		plasma aldosterone levels	282
	genetic predisposition (genetic polymorphisms)	BCHE, GPX1 and ROS1	283
		endothelial nitric oxide synthase	284
		platelet glycoprotein IIIa	285
		alpha-estrogen receptor	286
	other patient-depended risk factors	systemic arterial hypertension	281
		gender (female gender with a lower risk of restenosis after coronary stenting)	281,287
		elevated C-reactive protein	288-290
		recurrent restenosis	110
		earlier neointimal response and the extent of neointimal proliferation predicted a high risk for target vessel revascularization	291
		endothelial dysfunction (impaired flow-mediated dilation)	236,292
lesion- and procedure-dependent risk factors	vessel /lesion	lesion length (long lesions)	109,293
		lesion location (left anterior descending artery, ostial lesion)	294,295
		small vessel size	281,296
		plaque morphology (soft plaque)	297,298
		residual plaque burden	299
	stent	multiple stents	114,116
		longer stent length and physical stent parameters (thinner struts elicits less angiographic and clinical restenosis)	111,300-303
	procedure	postprocedural lumen diameter (smaller final minimal lumen diameter immediately after stenting)	111,114,295
		medial injury and lipid core penetration	143

with silent myocardial ischemia, peripheral artery disease, and stroke.[125] The exact pathogenetic mechanism(s) underlying development of DM-associated macrovascular disease are as yet unknown. Macrovascular disease in DM has been shown to be associated with a decline in EPC number and function which might therefore contribute to diabetic vascular complications.[126-129] Furthermore, the frequency of SMPCs was shown to be increased in subjects with Type 1 DM.[130] The data suggest that, as a result from DM, the balance between EPCs and SMPCs might be disturbed in favor of the latter thereby resulting in enhanced atherosclerosis and restenosis rates. This suggest the existence of

a causal relationship between the presence of DM, impeded capacity to restore vascular integrity, and the development of macrovascular disease.

Pathogenesis of ISR

ISR can be considered as a wound healing response[97] as the cellular and molecular mechanisms involved in ISR have similarities with those involved in wound healing.[112,131] During wound healing, best characterized by the healing response to dermal injury, a thrombotic and acute inflammatory reaction is followed by the granulation tissue phase with macrophage infiltration, myofibroblast influx and angiogenesis.[132] The events leading to ISR can be divided into two distinct phases based on the time of occurrence after stenting: the early phase (days to weeks) and the late phase (weeks to months).[112] The early-phase is characterized by endothelial damage caused by mechanical injury due to stent deployment followed by thrombotic events and inflammation.[112,133] The late phase is mostly associated with neointimal hyperplasia.[134] As shown by Farb *et al.* studying the course of the histopathological appearance of coronary arteries after stenting, early lesions are characterized by platelet-rich thrombus formation and an acute inflammatory response. In this early phase, neointima formation was absent in any of the patients with less than 11 days post-stenting follow-up. However, in the late phase neointimal lesions were present in 45% and 100% of patients with a post-stenting follow-up of 12-30 days and >30 days, respectively.[135]

In the development of ISR, neointima formation appears to precede reendothelialization. Early after stent deployment no ECs on the stented luminal surface are left. Even 6 weeks after stenting, the luminal border is not (fully) covered by ECs, and at this time neointimal VSMCs form the border between the neointima and the recirculating blood. Neointima formation starts with a reparative process characterized by coverage of the stented area by a thin, membranous thrombus with entrapped red blood cells, thrombocyte aggregates and polymorphonuclear leukocytes. Following acute endothelial denudation and platelet adhesion, in the absence of mature endothelium in the early phase, VSMCs accumulate and start to proliferate and secrete ECM components. Although 28 days after stenting some VSMCs can be detected in the developing neointima, after 12 weeks VSMCs form the main component of the neointima. Whereas reendothelialization is complete by 3 to 4 months, VSMC accumulation peaks around 9-18 months after stenting.[131,136-138]

There is an important relation between inflammation and ISR.[139-142] Stent-induced damage of the atherosclerotic plaques as well as the arterial wall (characterized by fibrous cap rupture, stent strut penetration into the lipid core and deep arterial laceration) is associated with (chronic) inflammation which is in turn associated with enhanced neointimal growth.[131,135,136,143,144] The stenting procedure itself forms a strong inflammatory stimulus, and ISR might therefore be regarded as a remodeling response to mechanical vascular damage induced by stent deployment.[94] Initial damage to ECs leads to up-regulation of endothelial adhesion molecules that will induce leukocyte recruitment followed by transmigration into the intima upon chemotactic stimulation with proinflammatory cytokines.[101] In addition to this local inflammatory response, stent deployment also elicits a systemic inflammatory

response which is characterized by increased circulating levels of pro-inflammatory mediators such as IL-1, IL-6 and TNF-α.[145] Following stenting, the accumulating platelets, leukocytes as well as the resident vascular wall cells (ECs and VSMCs) secrete cytokines and growth factors (*e.g.* PDGF, TGF-β, EGF) that in turn function as mitogens and chemotaxic agents for VSMCs and macrophages in a paracrine fashion[4,112,146,147] thereby aggravating the development of ISR.

Origin of neointimal cells in ISR

Alike TA, neointimal hyperplasia in ISR mainly consists of proliferating VSMCs[136,148,149] embedded in ECM.[134-136,142,150,151] As already outlined above for the development of TA, development of ISR is classically attributed to migration, proliferation and apoptosis of medial VSMCs[152] due to: 1) mechanical stretch, rupture of the internal elastic lamina, and medial dissection; 2) endothelial denudation with exposure to circulating mitogens such as angiotensin II and plasmin; and 3) release of mitogens and cytokines from platelets, ECs, medial VSMCs, and inflammatory cells.[102]

In line with the response-to-injury hypothesis proposed by Ross[62] for the development of atherosclerosis, neointimal VSMCs may originate from the media of the stented vessels. After stenting, medial VSMCs loose their quiescent contractile state and enter cell cycle. Medial VSMCs also start to migrate through the internal elastic lamina into the subendothelial space of the intima, where they start to synthesize ECM components and contribute to neointima formation.[136] According to this concept, medial VSMCs form a potential target for intervention by preventing dedifferentiation towards a synthetic phenotype characterized by migration, proliferation and ECM synthesis. In line with this, *in vivo* adenoviral delivery of A20 to medial VSMCs in rat carotid arteries was shown to attenuate medial VSMC proliferation and subsequent neointima formation following balloon angioplasty.[153]

However, as observed in TA accumulating evidence indicate a blood borne origin of neointimal cells also in different models of endovascular injury thereby raising the possibility that also sources other than the media (BM, circulation, peripheral tissues and vascular wall niches) provide progenitors cells for neointimal VSMCs after stenting. It is as yet unknown if, and to what extent, these cells contribute to neointima formation in ISR. However, scarce data suggest the contribution of BM-derived cells to neointima formation in ISR as Hibbert *et al.* showed the presence of c-kit$^+$ vascular progenitor cells in the neointima in human coronary ISR.[154] Moreover, Skowasch *et al.* have shown the presence of BM- and neural-crest-derived cells in atherectomy samples retrieved from patients with ISR also suggesting recruitment of extravascular cells that contribute to neointima formation after stenting.[23]

Transplant arteriosclerosis and in-stent restenosis: differences and similarities

As outlined in the previous sections above, vascular remodeling in TA and ISR appear to share common pathophysiological pathways, and may be both regarded as an abnormal healing

response to endovascular injury. Besides their aforementioned similarities (endothelial dysfunction, inflammation-driven, increased expression of EC adhesion molecules, and VSMC proliferation and migration), there are clear differences especially in the inciting events of vascular remodeling process as well as in the associated confounding factors that modulate their progression (Figure 2). Both the differential events as well as common pathways need to be considered when designing new therapeutic strategies to attenuate TA and ISR.

The various characteristics of TA and ISR are summarized in Table 2 in which differences and similarities are indicated. Endothelial dysfunction is an early feature of vascular disease[39] that contributes to the pathogenesis of both TA and ISR. Endothelial dysfunction reflects EC damage and is the first step in a cascade of pathological events that will end up in NI formation. Although the initial triggers of endothelial injury and dysfunction differ between TA and ISR (*i.e.* mechanical injury in ISR and transplantation-related endothelial injury in TA [see Table 2 and Figure 2]), the subsequent events following endothelial activation like leukocyte infiltration appear to be highly similar between the two vasculopathies. Moreover, both ISR and TA have deleterious effects on systemic EC function.[46,47,155,156] As endothelial dysfunction is a systemic disorder which is key in the pathogenesis of atherosclerosis and its complications[39], strategies aiming at improving systemic EC function will also translate into reduced cardiovascular morbidity and mortality.[157,158] Upon endothelial injury, (BM-derived) $CD34^+$ EPCs participate in

Table 2. Mechanisms and risk factors for TA and ISR: differences (red) and similarities (green)

Variable	Transplant arteriosclerosis (TA)	In-stent restenosis (ISR)
initiating injury	immune response	mechanical injury
time of development	months to years	months to years
localization	diffuse	focal
intimal proliferation	concentric	concentric
constrictive remodeling	present	absent
clinical manifestation	insidious	abrupt
EC injury	denuding and non-denuding	denuding
systemic endothelial dysfunction	present	present
predominant cellular mechanisms	EC damage, inflammation, thrombosis progenitor cell recruitment, VSMC migration and proliferation	EC damage, inflammation, thrombosis, progenitor cell recruitment, VSMC migration and proliferation
risk factors	Transplantation-dependent (acute rejection episodes, ischemia-reperfusion, donor brain death, major histocompatibility mismatch) and -independent (hyperlipidemia, diabetes, oxidative stress, hypertension, inflammation, elevated C-reactive protein, infections, environmental stimuli)	hyperlipidemia, diabetes, oxidative stress, hypertension, inflammation, elevated C-reactive protein, infections, environmental stimuli

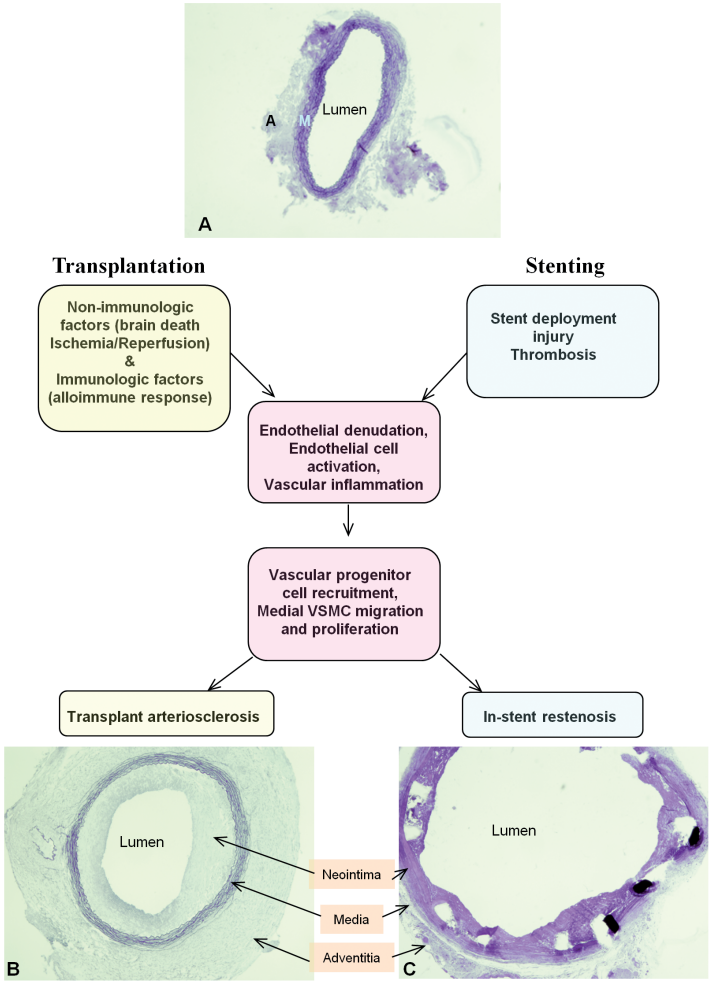

Figure 2. Pathobiology of transplant arteriosclerosis and in-stent restenosis. Although the initial triggers of endothelial injury and dysfunction differ between TA and ISR (*i.e.* mechanical injury in ISR and transplantation-related endothelial injury in TA), the subsequent events following endothelial activation (like leukocyte infiltration, vascular progenitor cell recruitment and proliferation) appear to be highly similar between the two vasculopathies. Representative photomicrographs after Lawson-staining (elastin) showing (A) a non-injured rat aorta, (B) neointima hyperplasia in rat aortic allograft (*i.e.* TA), (C) neointima hyperplasia in stented rat abdominal aorta. Abbreviations: A: adventitia; M: media.

reendothelialization.[159-162] Circulating endothelial progenitor cells are able to participate in vascular repair by homing to the injured vascular wall and differentiation into ECs (*i.e.* reendothelialization). In addition to these presumably beneficial effects of EPCs facilitating endothelial repair, recirculating (BM-derived) SMPCs may actually promote the development of coronary artery disease and restenosis.[11,14,160,163,164]

The inciting events leading to TA and ISR thus clearly differ (Table 2). Mismatched HLA-antigens on allografts act as chronic stimuli for ongoing inflammatory responses

(*i.e.* rejection) causing alloimmune-mediated damage to the vessel wall. After stenting, acute mechanical injury instead of rejection causes inflammation and results in chronic low-grade VSMC proliferation due to continuous presence of the stent.[94] Transplantation-related factors known to contribute to vascular injury in TA (*e.g.* acute rejection episodes, ischemia-reperfusion, donor brain death, HLA mismatch), are unique for allografted organs and are clearly absent in ISR. On the other hand there are also shared factors that play a role in both the development of TA and ISR such as hyperlipidemia, diabetes, oxidative stress, hypertension, cytokine modulation, inflammation, elevated C-reactive protein, infections, and other environmental stimuli such as smoking.[91,165]

Diabetes and hyperlipidemia

Diabetes is considered a major risk factor for ISR. Similarly, data from both experimental models[166] and clinical studies[167] suggest an important role for metabolic abnormalities also in the pathophysiology of TA. Hypertriglyceridemia, hyperhomocysteinemia, hypertension, hyperglycemia, obesity, and insulin resistance occur with a high frequency in heart transplant patients.[168,169] Hyperlipidemia is commonly seen in cardiac transplant patients and many of these patients were already hyperlipidemic even before they were transplanted. In addition, the immunosuppressive therapy given to transplant recipients (especially calcineurin inhibitors) may cause or exacerbate pre-existing dyslipidemia. This so-called metabolic syndrome is associated with endothelial dysfunction and atherosclerosis and development of occlusive vascular disease in the general population[170] and in particular in diabetics and heart transplant recipients.

Pre-existing atherosclerosis

Stenting is generally performed within a diseased artery with manifest atherosclerosis. Cardiac transplantation is performed using supposedly donor hearts non-affected by manifest atherosclerosis, although the endothelium might be damaged due to donor brain death and ischemia-reperfusion injury. Nevertheless, clinical studies also suggest a role of donor-transmitted, focal non-circumferential atherosclerotic plaques in the development of TA in heart transplant recipients.[171] Although pre-existing donor lesions do not directly act as a starting point for the development of TA and do not affect survival of recipients, patients that received grafts with pre-existing atherosclerosis have a higher incidence of angiographic TA.[172]

VSMC proliferation and migration

Cytokine-induced activation of VSMCs is a critical cellular event in the development of both TA and ISR.[91,173] The key stimuli for intimal hyperplasia that have been defined are injury, inflammation and increased mean wall stress, and these are all present in both TA and ISR and culminate in a final common pathway of VSMC proliferation and migration.[4]

Although luminal narrowing is concentric both in TA and ISR (Figure 2 & 3), there are differences with regard to the extent of the lesions and their location. In TA neointima formation is diffuse, with both major epicardial vessels and intramyocardial vessels being

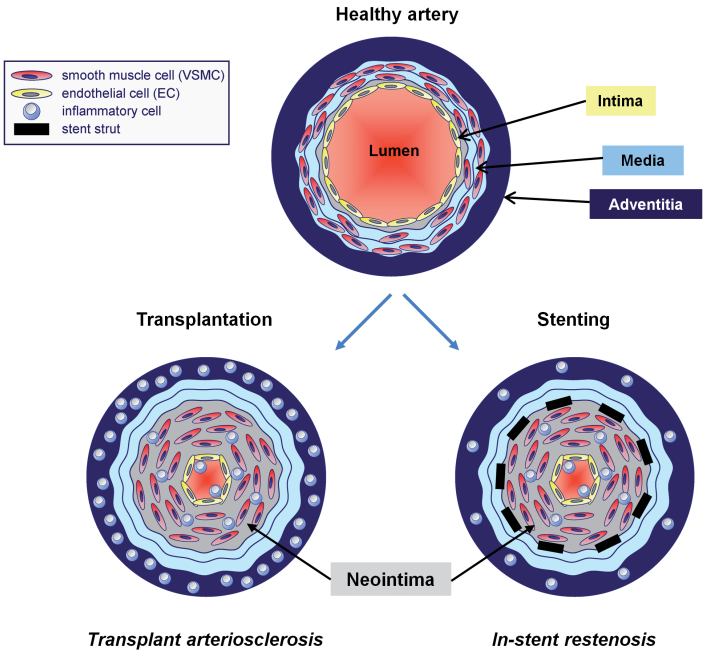

Figure 3. Neointima formation is the pathological hallmark of transplant arteriosclerosis and in-stent restenosis. This figure schematically displays arterial cross-sections of a healthy artery and arteries with transplant arteriosclerosis and in-stent restenosis. Upon vascular injury (alloreactivity against graft in transplantation and mechanical injury caused by stent struts) recruitment, homing and proliferation of vascular smooth muscle cells will result in intimal thickening and lumen narrowing.

affected in cardiac transplantation. Conversely, in ISR the neointima is focal, and present at the stented region only. However, in contrast to circumferential neointima formation, which is more common in smaller arterial segments in TA, also focal plaque formation might appear in larger coronary arteries.[171] Also after stenting concentric narrowing of the lumen is usually observed, although a more focal neointimal thickening can be observed which is associated with increased intramural bleeding.[136] Constrictive remodeling has been described in TA.[174-176] Although in ISR constrictive remodeling has a minimal importance due to the presence of the wire stent, there is a gradual reduction in neointima between 6 months and 3 years,[177] suggesting that the neointima itself can remodel over time.[94]

Kinetics of TA and ISR development

Difference between ISR and TA can also be observed regarding the kinetics of progression and severity. TA generally manifests after the first year post-transplantation with insidious clinical features culminating in graft failure eventually. ISR is characterized by an abrupt onset and rapid progression with intense proliferative activity followed by either stabilization or sudden appearance of a brutal ischemic event requiring urgent surgical intervention to restore perfusion. However, use of intravascular ultrasound (IVUS) revealed abnormal intimal thickening in 50% of transplant recipients already at 1 year post transplantation.[178]

Stenting is demonstrated to have a triphasic luminal response[179]: the early restenosis phase (until 6 months) and the intermediate-term regression phase (from 6 months to 3 years) are followed by a late renarrowing phase beyond 4 years, when progressive increase in the incidence of late target lesion revascularization was observed up to 15 to 20 years after bare metal stent implantation.[180]

Therapeutic perspectives to attenuate TA and ISR

Therapeutic strategies to attenuate TA

Although the use of improved immunosuppressive agents has resulted in adequate prevention and treatment of acute rejection[181,182], adequate curative or preventive treatment of the development of TA is still lacking thereby remaining retransplantation as the only effective therapy. Besides efforts to optimize immunosuppressive therapy, attention has been directed towards disease prevention via attenuation of adverse non-immunologic and immunologic reactions.[41,181,183,184] Once TA is established, pharmacological treatment options are limited and non-pharmacologic interventions are preferred.[181] Fibrofatty plaques and focal coronary lesions are also displayed in TA[185] which may be suitable for percutaneous coronary intervention and occasionally coronary bypass surgery. Therefore, stenting is a palliative cardiac interventional procedure applied both in native atherosclerosis and in TA, although interventions for revascularization in transplanted organs usually have disappointing short- and medium-term outcomes.[186-188]

Maintaining endothelial integrity and inhibiting VSMC proliferation represent two major goals in controlling NI formation. As EC injury is the first and most important trigger of TA development after cardiac transplantation[189], rapid recovery from graft EC loss is key in preventing further graft EC injury and TA. The extent of endothelial cell apoptosis was directly correlated with the subsequent fibroproliferative changes.[190-192] Moreover, the magnitude of EC chimerism was associated with vascular rejection thereby reflecting more aggressive alloreactivity against graft endothelium.[193] Therefore, besides enhancing reendothelialization, preserving the graft endothelium seems to be a more rational approach to limit NI formation. Various (experimental) therapeutic approaches have been applied to improve EC function and reduce activation following transplantation[194] as well as to enhance reendothelialization of mechanically injured vessels.[195] These EC-directed therapies include mobilization of EPCs by pharmacologic treatment with *e.g.* statins[196], cytokine-induced mobilization of hematopoietic precursor cells[197], cell transfer of EPCs that are obtained from different sources[198-200], overexpression of endothelial nitric oxide synthase (eNOS) in EPCs[201], drug-eluting stents coated with an integrin-binding cyclic Arg-Gyl-Asp peptide or an antibody against CD34 which were able to capture circulating EPCs.[202,203]

Several drugs other than statins are able to increase the number and functional activity of EPCs including peroxisome-proliferator activated receptor-γ (PPARγ) agonists (thiazolidinediones), angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (Arbs), and erythropoietin.[204-206] In studies in which EPC availability was

therapeutically enhanced, reduced NI formation as well as improved vascular wall function were commonly observed after endovascular injury (reviewed in [162,195]). These observations clearly indicate that in restenosis, therapeutically enhanced reendothelialization is indeed beneficial. However, whether the same holds true for the development of TA still remains to be elucidated. In the view of therapeutic strategies, one should at least take into account one major difference between the development of TA and restenosis, *i.e.* the duration of the damage-inducing insults. In restenosis, there is only one major insult that induces damage that needs to be repaired (balloon inflation and the stenting procedure) with a persistent peri-strut chronic inflammatory response due to a foreign-body inflammatory reaction to the metal struts[207], whereas after allografting, the intragraft vasculature is continuously exposed to damage-inducing factors due to ongoing subclinical rejection.

Caution is however warranted as, in contrast to some positive effects on reendothelialization, other data suggest that EPCs may actually promote TA rather than having a favorable effect on neointima development. After allogeneic aorta transplantation in BM-chimeric mice with Tie2-LacZ BM (mice expressing β-galactosidase in ECs) Hu *et al.* observed vasculogenesis within the neointimal lesions characterized by microvessel formation consisting of BM-derived ECs. Neointimal vasculogenesis developed in addition to neointimal EC replacement with non-BM-derived ECs.[208] Microvessels appeared in allografts even before the onset of neointimal formation, suggesting that vasculogenesis within the intima may be a crucial event for the development of TA. If this dual role of circulating endothelial cells in the development of TA holds true, future strategies should on the one hand aim at enhancing reendothelialization with non-BM-derived circulating ECs, and on the other hand impair EPC-mediated vasculogenesis within the intima. In addition to neointimal vasculogenesis, neovascularization in the adventitia was shown to be responsible for promotion of intimal thickening[209] and a correlation between adventitial neovascularization and aortic plaque progression in apolipoprotein E-deficient mice was shown.[210] Inhibition of new *vasa vasorum* formation in the adventitia in response to injury might reduce circulating vascular progenitor cell recruitment. Moreover, a bipotential differentiation capacity of BM- and periphery-derived angiogenic progenitor cells has been demonstrated *i.e.* the capacity to differentiate into both EC and VSMC lineages.[83] Therefore, therapeutic manipulation to skew differentiation of a common angiogenic ancestral progenitor cell towards the endothelial lineage may attenuate restenosis after angioplasty.[83] Further studies are required in order to determine which factors actually drive angiogenic progenitor cell differentiation towards the EC and VSMC lineage.

In addition to ECs, VSMCs are the major players in NI development and therefore their recruitment, migration and proliferation form potential targets for therapeutic intervention. Some of the currently used immunosuppressive drugs (including rapamycin, mycophenolic acid, cyclosporin, calcium channel blockers, and statins) have been shown to possess anti-proliferative effects on VSMCs.[61] Proliferation signal inhibitors like sirolimus and its derivative everolimus were indeed associated with lower rates of acute rejection and reduced development of TA.[211,212] Moreover, experimental interventions to modulate cell cycle progression[6,213] and induction of expression of protective genes in the vessel wall[214-216]

have been used to modulate the molecular programs that underlie medial VSMC migration and proliferation. However, strategies as such have not been demonstrably able to abolish TA development. The therapeutic measures might have higher efficacy when they target the SMPCs before their homing and proliferation is mounted, thereby interfering in early events. Modalities to influence their mobilization, recruitment, homing, and differentiation might represent efficacious strategies to limit NI formation. Cytokines and chemokines that have been shown to be important in these steps include granulocyte colony stimulating factor (G-CSF), stromal derived factor-1α (SDF-1α), c-kit and c-kit ligand (KitL, also known as stem cell factor or SCF), matrix metalloproteinase-9 (MMP-9), and vascular endothelial growth factor (VEGF) and its receptor.[161] Among them, the SDF-1α/CXCR4 axis has been reported to be involved in progenitor cell recruitment in numerous injury models including TA and its blockade resulted in decreased intimal hyperplasia.[217-221]

As current immunosuppressive regimens are still rather ineffective in preventing TA, other approaches will be needed. Instead of focusing only on eliminating the 'weapon' by debilitating the immune system, also protection of the target (the grafted organ) might help to control NI formation. In summary, several strategies can be identified, including 1) preserving the endothelial lining and selectively increase EPC homing to promote reendothelialization, 2) blocking inflammatory and proliferative responses to impede VSMC migration and proliferation, and 3) limiting the recruitment and homing of SMPCs into the graft vessels. Future studies are needed in order to demonstrate whether approaches as such, in adjunct to current immunosuppressive protocols, are able to attenuate TA development.

Therapeutic strategies to attenuate ISR

The aforementioned potential strategies for limiting NI hyperplasia in TA by enhancing reendothelialization through EPC mobilization, recruitment and differentiation on the one hand, and inhibiting SMPC mobilization, recruitment, differentiation and proliferation on the other, is potentially valid also for prevention of ISR. Besides these common therapies of selective modulation of progenitor cell subsets and inhibiting VSMC proliferation, also some specific therapeutic approaches to target ISR can be identified.

Development of ISR involves a cascade of traumatic, thrombotic, proliferative and late remodeling phases, culminating in the accumulation of VSMCs and matrix components in the NI eventually.[151,222] Given the role of inflammation in ISR, research in vascular biology has focused on strategies to modulate the inflammatory process in the vessel wall. Various studies demonstrated that treatments that inhibited inflammation and reduced the expression of inflammatory cell adhesion molecules have beneficial effects on the development of ISR.[223-225] For example, blockade of early monocyte recruitment resulted in reduced neointimal hyperplasia after stenting.[223,224,226] Furthermore, clinical studies have shown reduced ISR with sirolimus- and paclitaxel-coated stents; agents with both anti-proliferative and anti-inflammatory effects.[227-229] Also PPARγ agonists (thiazolidinediones), which are known to have anti-inflammatory properties[230], have been shown to attenuate intima hyperplasia after coronary stenting in both diabetic and non-diabetic patients.[231-234]

Alike TA development, the presence of intact endothelium in mechanically injured arteries suppresses neointimal hyperplasia.[196,198-200] The status of the vascular wall, and especially the vascular endothelium, is the key component of the response to vascular injury.[235] Stenting damages the endothelium thereby resulting in endothelial dysfunction which is the result of an imbalance between vasodilator substances with anti-proliferative properties (such as NO), and vasoconstrictors with mitogenic properties (such as endothelin).[39] Patti *et al.* showed that impaired flow-mediated dilation of the brachial artery independently predicts occurrence of coronary ISR.[236] The systemic and local milieu associated with endothelial dysfunction favors cell proliferation, intimal hyperplasia, and vasoconstriction, which may all contribute to the development of restenosis. Indeed, local release of endothelin-1 at the site of vascular injury has been observed[237], and treatment with an endothelin receptor antagonist attenuates ISR.[238] Moreover, the administration of L-arginine, the precursor of nitric oxide (NO), inhibits lesion formation after balloon injury[239] and improves coronary EC function.[240] The recovery of the vascular endothelium and the restoration of EC function early after revascularization are important therapeutic targets for the prevention of restenosis and associated clinical events.[235] Stented arteries with intact endothelium favors for a faster reendothelialization and less intimal thickening compared with previously denuded stented arteries.[241] Moreover, perivascular implantation of tissue-engineered ECs around injured arteries has been shown to reduce neointimal thickening in bovine and porcine models of carotid arterial injury, thereby underscoring the critical role of ECs in the regulation of vascular function and vascular remodeling.[242,243] Many of the protective actions of the endothelium is mediated by NO which was shown to inhibit VSMC migration and proliferation.[244] In line with this, NO-eluting stents are able to significantly reduce ISR in rabbits.[245] Moreover, local gene transfer of inducible nitric oxide synthase (iNOS) was shown to reduce NI formation in pigs.[246,247] Therefore, increasing bioavailability of NO and NOS might represent potential therapeutic approaches to control vascular healing. Besides local interventions, therapies with drugs that improve EC function (such as ACE inhibitors, statins, and antioxidants) have been shown to reduce the risk of restenosis in patients with EC dysfunction.[39,45,158,236]

The two major complications associated with stenting are stent thrombosis and restenosis.[91] Initially, thrombotic events were responsible for the high rate of loss of stent patency early after stenting. However, changes in antiplatelet therapy[248] and improvement of stent implantation techniques have significantly decreased the incidence of fatal stent thrombosis to less than 1%.[222] Although the thrombotic process can be almost entirely prevented, its inhibition does not ensure long-term vessel patency[222] as stenting still results in a relatively high restenosis rate.

Table 3 summarizes the various therapeutic approaches that have been tested for their efficacy in reducing restenosis, including mechanical angioplasty devices (reviewed in [91,101,249]), brachytherapy (intracoronary radiation)[250-252], gene therapy[246,247,253-255], local[256-260] and systemic[101,261-263] drug therapy, and the use of drug-eluting stents.[202,227-229,252,264-270] Among these treatments, especially intravascular brachytherapy and the use of rapamycin- or paclitaxel-drug-eluting stents had a significant impact on reducing ISR.[228,229,250-252,264,271] Brachytherapy

Table 3. Pharmacological and cellular therapies to prevent restenosis

Type of intervention	Reported interventions	References
mechanical angioplasty devices to remove neointima	percutaneous transluminal coronary angioplasty, directional coronary atherectomy, high speed rotational atherectomy, cutting ballon	reviewed in 91,101,249
brachytherapy	intra-luminal radiation results in a lower rate of ISR	250-252
gene therapy	gene-delivery of anti-MCP-1 gene reduced ISR in rabbits and monkeys	254
	acceleration of reendothelialization via VEGF-2 gene-eluting stents inhibits neointimal proliferation in a hypercholesterolemic rabbit iliac angioplasty model	304
	inducible nitric oxide synthase gene transfer was shown to reduce NI formation in pigs	246,247
local drug therapy	paclitaxel delivered into the adventitia of pig femoral arteries effectively attenuates neointima after angioplasty	257
	treatment of coronary ISR with paclitaxel-coated balloon catheters in humans reduced the incidence of restenosis	256,258-260
systemic drug therapy	antithrombotic	reviewed in 101,261
	antiinflammatory, antiproliferative: oral adjunctive sirolimus treatment after stent implantation results in significant improvement in the angiographic and clinical parameters of restenosis	262,263
	peroxisome proliferator-activated receptor gamma agonists	231,305,306
drug-eluting stents	antiproliferative and antiinflammatory proprieties (sirolimus, paclitaxel, everolimus, zotarolimus)	144,227-229,252,264-268,270
	enhancing reendothelialization (capture circulating EPCs): stents coated with an integrin-binding cyclic Arg-Gly-Asp peptide in porcine coronary arteries, stents coated with an antibody against CD34 in humans	202,203
	increasing nitric oxide bioavailability (NO eluting stents significantly reduce ISR in rabbits)	245

results in a 50% reduction in restenosis and clinical events compared with repeated balloon angioplasty but features late thrombosis.[250,251] Drug-eluting stents have reduced angiographic restenosis rates by ≥75% compared with bare-metal stents.[271,272] Nonetheless, drug-eluting stents still do not completely solve the problem of ISR as the antiproliferative drug induces a cytostatic or cytotoxic effect on the neointimal vascular tissue which also impedes the natural healing response by delaying the formation of a functional endothelial layer covering the stent. Therefore, drug eluting stents hamper the reendothelialization process as compared with bare metal stents, which correlates with late stent thrombosis.[137,273-275] An inhibiting effect of sirolimus on circulating vascular progenitor cells, leading to inhibition of NI formation, but also to a retarded reendothelialization was demonstrated.[276] This observation supports the idea that sirolimus might interfere with vascular healing response. It seems that the prevention of one iatrogenic disease (ISR) produces another iatrogenic disease (late stent thrombosis), which requires a further level of treatment/prevention for its solution.[277,278]

Although systemic or local delivery of anti-proliferative drugs like rapamycin or paclitaxel reduces ISR in humans[258,262,263], development of ISR was not completely prevented. Therefore, a treatment strategy solely based on anti-proliferative actions may not be sufficient to completely block development of ISR. Cell migration rather than proliferation was shown to contribute to NI formation in the early phase after stenting. This stresses the importance of early therapeutic intervention with especially anti-migratory compounds after stenting[279], and targeting of recruitment signals like the SDF-1α/CXCR4 axis and the SCF/c-kit pathway.[221,280] The multifactorial nature of the response-to-injury argues against manipulation of a single biological process. Treatments to prevent ISR would ideally consists of a combinatorial approach using agents that reduce thrombosis and inflammation and enhance reendothelialization (by modulating angiogenic progenitor cell recruitment and differentiation), inhibit medial VSMC migration and neointimal cell proliferation by targeting multiple cell cycle check points and reduce ECM synthesis.[277]

Prevention of neointimal hyperplasia appears to require a multibranched approach and therapies aiming at reducing NI formation should interfere at different levels in the arteriosclerotic process, as one magic "bullet" that blocks all pathological events contributing to ISR is unlikely to exist.

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2

Introduction to the experimental work

The aim of the studies presented in this thesis is to investigate various cellular and molecular aspects of vascular remodeling processes following solid organ transplantation (*i.e.* transplant arteriosclerosis [TA]) and endovascular stenting (*i.e.* in-stent restenosis [ISR]) thereby focusing on ECs, VSMCs and their progenitors. For the development of TA the role of VSMCs and their putative progenitors on NI formation as well as new potential therapeutic approaches to simultaneously reduce alloreactivity and modulate neointimal cell (ECs and VSMCs) homing and proliferation were studied (Chapters 3-5). For the development of ISR blood-borne *vs.* BM origin of neointimal cells and the influence of diabetes on this process were investigated (Chapters 6-8).

TA is the major cause of long-term allograft loss and mortality in heart transplant recipients. In the clinical setting there is variation in both the rejection rate and long-term outcome among transplant recipients, transplanted with similar HLA-incompatible grafts and receiving similar immunosuppression. This variation suggests a genetic susceptibility for TA development which has not been fully explained. In **Chapter 3** we studied the rate of TA development in rat aortic allografts transplanted in different rat strain combinations and analyzed the possible underlying cellular and molecular mechanisms involved in causing differential TA development. To this end, the proliferative capacity of neointimal VSMCs (which were previously shown to be recipient-derived in this model), and the kinetics of reendothelialization and VSMC progenitor cell mobilization were analyzed. TA development is refractory to conventional immunosuppressive drugs and adequate therapy is not available. Synthetic PPARγ agonists (belonging to the family of thiazolidinediones) are known to possess anti-inflammatory properties and have been shown to attenuate intima hyperplasia after coronary stenting. In **Chapter 4** the effect of the thiazolidinedione rosiglitazone on TA development was analyzed as well as its effects on T cell alloreactivity, neointimal VSMC proliferation, and expression of progenitor cell recruitment factors. Since the transplant-associated inflammatory state is associated with systemic EC dysfunction (outside the graft), in **Chapter 5** the effects of rosiglitazone on systemic EC function and vasomotor activity after transplantation were investigated.

ISR is the most common, life-threatening, complication following stenting. Although stenting results in reduced restenosis rates as compared to percutaneous transluminal coronary angioplasty, ISR remains a clinical problem for which adequate treatment is still lacking. The anatomical source of neointimal VSMCs in ISR is as yet unknown. Elucidation of their origin might offer possibilities to design new treatment modalities to target neointima formation already in the early phase following stenting. In **Chapter 6** we studied the origin of neointimal VSMCs in experimental ISR and determined the contribution of BM-derived cells in ISR. Diabetic patients have a higher frequency of ISR as compared to non-diabetics. The underlying mechanisms of this effect of diabetes is still not fully clear. Research on the mechanisms of diabetes-enhanced ISR is hampered by the lack of a suitable animal model. We therefore developed a novel long-term diabetic rat model with poor glycemic control as described in **Chapter 7**. In this model the effect of long-term hyperglycemia on ISR after aortic stenting was studied. Diabetes is associated with disturbed neovascularization with binary outcome: reduced neovascularization (as observed in revascularization of ischemic

tissue, impaired wound healing, embryonic vasculopathy, and organ transplant rejection) or enhanced neovascularization (as observed in diabetic retinopathy, diabetic nephropathy, and possibly atherosclerotic plaque destabilization). Both resident endothelial cells as well as circulating endothelial progenitor cells may contribute to neovascularization processes. In **Chapter 8** we studied the effect of diabetes on angiogenic sprout formation from the vessel wall as a reflectant of the angiogenic potential mediated by pre-existing vessels or local resident progenitor cell niches. In **Chapter 9** a summary of the results described in this thesis is presented and the obtained data are discussed in a broader context. This chapter concludes with some future perspectives: what have we learned from the studies described in this thesis and in which direction do we need to go with future research.

3

Development of transplant vasculopathy in aortic allografts correlates with neointimal smooth muscle cell proliferative capacity and fibrocyte frequency

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Abstract

Objective. Transplant vasculopathy consists of neointima formation in graft vasculature resulting from vascular smooth muscle cell recruitment and proliferation. Variation in the severity of vasculopathy has been demonstrated. Genetic predisposition is suggested as a putative cause of this variation, although cellular mechanisms are still unknown. Using a rat aorta transplant model we tested the hypothesis that kinetics of development of transplant vasculopathy are related to neointimal smooth muscle cell proliferative capacity and fibrocyte frequency, the latter being putative neointimal smooth muscle ancestral cells.

Methods. Aortic allografts were transplanted in Lewis and Brown Norway, as well as MHC-congenic Lewis.1N and Brown Norway.1L recipients. Severity of transplant vasculopathy was quantified 4, 8, 12 and 24 weeks after transplantation. Host-endothelial chimerism, as a reflection of vascular injury, was determined by specific immunofluorescence. Neointimal smooth muscle cell proliferative capacity was determined *in vitro* and *in situ*. Fibrocyte frequency and phenotype were determined after *in vitro* culture by cell counting, immunofluorescence and *in situ* zymography.

Results. Compared to Lewis, Brown Norway recipients developed accelerated transplant vasculopathy which is dependent on the presence of Brown Norway non-MHC-encoded determinants. Accelerated transplant vasculopathy was associated with increased levels of host-endothelial chimerism and increased neointimal smooth muscle cell proliferation, the latter being accompanied by increased Endothelial and Smooth Muscle Cell-derived Neuropilin-like Protein mRNA expression. Moreover, accelerated transplant vasculopathy was associated with increased frequency of circulating gelatinase-expressing $CD45^{+}vimentin^{+}$ fibrocytes.

Conclusion. Susceptibility for transplant vasculopathy appears to be genetically controlled and correlates with neointimal smooth muscle cell proliferative capacity and circulating fibrocyte frequency.

Abbreviations

α-SMA	smooth muscle α-actin	**MMP**	matrix metalloproteinase
BN	Brown Norway	**mSMC**	medial smooth muscle cell
CTD	chronic transplant dysfunction	**NI**	neointima
DA	Dark Agouti	**niSMC**	neointimal smooth muscle cell
EC	endothelial cell	**PDGF-BB**	platelet-derived growth factor-BB
ESDN	endothelial and smooth muscle cell-derived neuropilin-like protein	**PBMC**	peripheral blood mononuclear cell
HLA	human leukocyte antigen	**SMC**	smooth muscle cell
Lew	Lewis	**TV**	transplant vasculopathy
MHC	major histocompatibility complex		

Introduction

Development of chronic transplant dysfunction (CTD) is the primary cause of loss of solid organ transplants after the first postoperative year. Adequate strategies to prevent or treat CTD are still lacking.[1] The common histologic feature of CTD is transplant vasculopathy (TV) which is characterized by a generalized, concentric intimal thickening predominantly consisting of mesenchymal cells intermingled with inflammatory cells.[2,3] Neointimal mesenchymal cells are considered as vascular smooth muscle cells (SMCs) and are generally identified based on the expression of smooth muscle α-actin (α-SMA) which is, however, also expressed by myofibroblasts. TV has generally been accepted as the main cause of progressive deterioration in graft function.

Among transplant recipients, transplanted with similar HLA-incompatible grafts and receiving similar immunosuppression, variation exists in both the rejection rate and the long-term outcome. This variation is not fully explained but may to some extent result from differences in preservation, ischemia-reperfusion, and surgical trauma. Moreover, data indicate that different individuals might display different immune responses against an allograft (*i.e.* a different immunologic responder-status) which is likely to affect long-term outcome.[4] Possible candidates responsible for such individual variation are genetic variance in the regulation of cytokine and growth factor gene expression.[5-8] However, despite these correlations between differential gene expression and allograft outcome, cellular mechanism(s) underlying kinetics of TV development are as yet unknown.

In order to analyze the cellular mechanism(s) underlying kinetics of TV development, we evaluated the contribution of recipient MHC- and non-MHC-encoded determinants in the development of TV. Therefore, we analyzed the development of TV after aorta transplantation in various rat strain combinations. Since we previously showed that neointimal SMCs (niSMCs) and endothelial cells (ECs) in aortic allografts are all host-derived[9] and presumably blood-borne[10] we tested the hypothesis that TV responsiveness is associated with intrinsic niSMC proliferative capacity and circulating fibrocyte frequency, the latter being putative niSMC ancestral cells.

Our results show that susceptibility to develop TV in rat aortic allografts is associated with recipient non-MHC-encoded determinants that may influence niSMC proliferative capacity and circulating fibrocyte frequency.

Materials and Methods

Rats

Male Lewis (Lew), Brown Norway (BN), and Dark Agouti (DA) rats were obtained from Harlan (Zeist, the Netherlands). MHC-congenic Lewis.1N (Lew.1N) and Brown Norway.1L (BN.1L) rats were obtained from the Central Animal Facility of the Maastricht University Medical Center. Rats (8-12 wks of age) were kept under clean conventional conditions and were fed standard rat chow and acidified water *ad libitum*. All animals received humane care

in compliance with the Principles of Laboratory Animal Care (NIH Publication No.86-23, revised 1985) and the Dutch Law on Experimental Animal Care.

Experimental groups

To study differences in kinetics of TV development, aortic transplantation was performed in different rat strain combinations and sacrificed at various time-points after transplantation as listed in Table 1. Development of TV was determined in the fully MHC-incompatible BN-to-Lew (groups 1-4) and Lew-to-BN (groups 5-8) strain combinations. BN isografts (group 9) served as controls (Table 1A). To study the relative contribution of recipient's MHC- and non-MHC-encoded determinants, allografting was performed in MHC-congenic Lew.1N and BN.1L recipients (Table 1B). Congenic Lew.1N rats carry BN-MHC on the Lew non-MHC background, whereas congenic BN.1L rats carry Lew-MHC on the BN non-MHC background. The following combinations were included: BN-to-BN.1L (groups 10,11) and Lew-to-Lew.1N (groups 12,13) (MHC-incompatible but non-MHC identical) as well as DA-to-BN.1L (group 14) and DA-to-Lew.1N (group 15) (both MHC- and non-MHC-incompatible). The correlation between circulating fibrocyte frequency, host EC-chimerism and TV-responder-status was analyzed in the BN-to-Lew (groups

Table 1. Experimental groups used for aorta transplantation

	Group	N	Donor strain	Recipient strain	MHC disparity	non-MHC disparity	Sacrifice [a]
A	1	8	BN	Lew	+	+	4
	2	4	BN	Lew	+	+	8
	3	4	BN	Lew	+	+	12
	4	4	BN	Lew	+	+	24
	5	8	Lew	BN	+	+	4
	6	4	Lew	BN	+	+	8
	7	4	Lew	BN	+	+	12
	8	4	Lew	BN	+	+	24
	9	4	BN	BN	-	-	12
B	10	4	BN	BN.1L	+	-	4
	11	4	BN	BN.1L	+	-	8
	12	4	Lew	Lew.1N	+	-	4
	13	4	Lew	Lew.1N	+	-	8
	14	6	DA	BN.1L	+	+	4
	15	6	DA	Lew.1N	+	+	4
C	16	5	BN	Lew	+	+	1
	17	5	BN	Lew	+	+	2
	18	5	Lew	BN	+	+	1
	19	5	Lew	BN	+	+	2

[a] wks after transplantation

16,17) and Lew-to-BN (groups 18,19) combinations (Table 1C). Grafts were processed for histological and morphometric analyses (groups 1-15) and additionally analyzed for the presence of host-EC chimerism (groups 16-19).

Aorta transplantation

The abdominal aorta between the left renal artery and the bifurcation was removed from the donor and perfused with saline. Subsequently, the aortic graft (10-12 mm) was orthotopically transplanted via end-to-end anastomosis using 9-0 nylon suture. Total cold ischemic time was consistently <25 minutes during which the graft was kept in ice-cold saline. Recipient rats did not receive anti-rejection therapy.

Quantification of transplant vasculopathy

Grafts removed at autopsy were fixed in Bouin's fixative and embedded in paraffin. Tissue sections (7 μm) were taken from the center of each graft and were stained with Lawson's solution (Klinipath) to visualize elastic laminae. TV was quantified using a computerized morphometric analysis system (QWin Software, Leica Microsystems). Surface neointima was quantified in 6 sections from each graft: 3 sections cut at the middle of the graft and 3 sections cut at least 100 μm further into the graft. Surface neointima was measured in six sections by subtracting lumen area from internal elastic lamina area and the mean surface neointima in each graft was calculated.

Evaluation of host EC-chimerism in aortic allografts

Host EC-chimerism was determined in grafts transplanted in the Lew-to-BN and BN-to-Lew combinations explanted 1 and 2 wks after transplantation. Cryosections (5 μm) were stained using dual immunolabeling with endothelial-specific (clone HIS52/RECA-1) and BN MHC-class I-specific (clone OX27) mAbs in order to determine the origin (graft *vs.* recipient) of the ECs at the luminal side of the graft. The technical procedure for dual immunolabeling is described in detail in *Supplementary Data*.

Rat medial and neointimal SMC isolation, culture and phenotyping

For niSMC isolation, abdominal aorta transplantations were performed in Lew-to-BN and BN-to-Lew combinations. Grafts were explanted 8-10 wks post-transplantation. For medial SMC (mSMC) isolation, freshly collected thoracic aortas were used. Aortic allografts and thoracic aortas were opened longitudinally after which the endothelium was removed using a cotton swab. In allografts, the neointima was dissected from the media using foreceps. In thoracic aortas, the tunica media was dissected from the adjacent adventitia. Neointima and media were cut into small fragments and enzymatically digested as described in detail in *Supplementary Data*. Both mSMCs and niSMCs were cultured in SMC growth medium and phenotyped using immunofluorescence as described in detail in *Supplementary Data*.

Neointimal SMC proliferation in vitro

niSMCs were plated in 96-well flat bottom culture plates in SMC growth medium (EMEM supplemented with 4 mM glutamine, 100 mM NEAA, 10% FBS and 50 µg/mL gentamycin) at a density of $1.5x10^4$ cells/well. Plated cells were allowed to attach for 24 hrs. Cells were then growth-arrested for 72 hrs in SMC starvation medium (containing 0.4% FBS). Subsequently, cells were stimulated for 48 hrs with SMC stimulation medium (containing 40% FBS). Both early and late passages were used (between passage 4 and 27) with similar results. After adding 0.5 µCi/well [^{3}H]-thymidine for the last 24 hrs of culture, cells were trypsinized and harvested on glass filters. [^{3}H]-thymidine incorporation was assessed by liquid scintillation and results were expressed as disintegrations per second (dps).

Neointimal SMC proliferation in situ

To detect proliferating cells in Lew-to-BN and BN-to-Lew aortic allografts (harvested 4 weeks after transplantation), immunohistochemistry for Ki67 expression was performed on 5 µm 2% paraformaldehyde-fixed cryosections. Sections were permeabilized in 0.5% TritonX-100 followed by endogenous peroxidase blockade using 0.075% H_2O_2 in PBS. After rinsing in PBS, sections were incubated with rabbit-anti-human Ki67 polyclonal antibody (NCL-Ki67p, Novocastra™) for 1 hour. After rinsing in PBS sections were sequentially incubated with goat-anti-rabbit-HRP and rabbit-anti-goat-HRP (both from Dako). HRP-activity was visualized using 3-amino-9-ethylcarbazole and nuclei were counterstained with Mayer`s hematoxylin. Sections were coverslipped in Kaiser's glycerol-gelatin and analyzed on an Olympus BX50 research microscope equipped with AnalySIS software (Soft Imaging System).

To determine SMC or inflammatory cell phenotype of proliferating cells, dual immunofluorescent labeling was performed on 5 µm 2% paraformaldehyde-fixed cryosections using anti human-Ki67 rabbit polyclonal antibody combined with antibodies against CD45 (clone OX1, mIgG1, tissue culture supernatant) or α-SMA (clone 1A4, mIgG2a, Dako). After fixation, endogenous biotin was blocked (Biotin Blocking System, Dako) followed by incubation with 10% normal goat serum for 15 min. Sections were then incubated with primary antibodies (anti-Ki67/anti-α-SMA or anti-Ki67/anti-CD45) for 1 hour at room temperature. Primary incubation was followed by 30 min incubation with biotin-conjugated goat-anti-rabbit (Dako) and Alexa Fluor488-conjugated goat anti-mIgG2a or Alexa Fluor488-conjugated goat anti-mIgG1 (both from Invitrogen-Molecular Probes) polyclonal antibodies. Biotin was visualized using streptavidin-Alexa Fluor555. Nuclei were counterstained with DAPI and slides were coverslipped in Aqua Polymount. Sections were analyzed using a Leica DMRXA fluorescent microscope and pictures were taken using Leica Qwin software (Leica Microsystems).

Real-time RT-PCR analysis

mRNA expression levels of Endothelial and Smooth Muscle Cell-derived Neuropilin-like Protein (ESDN), platelet-derived growth factor (PDGF)-BB and its receptors PDGFRα and PDGFRβ in niSMCs were determined using real-time RT-PCR analysis. RNA isolation,

cDNA synthesis, primer sequences and product size, and real-time RT-PCR are described in detail in *Supplementary Data*.

Quantification of the frequency of circulating fibrocytes

To determine the frequency of circulating fibrocytes, aorta transplantations were performed in the Lew-to-BN and BN-to-Lew combinations. PBMCs were isolated before transplantation, and 7 and 14 days after transplantation and cultured for 7 days after which the number of spindle-shaped cells was counted. Numbers of fibrocytes are expressed as the percentage adherent cells of the total number of plated cells or as the percentage elongated cells of the total number of adherent cells. Technical details of PBMC isolation, culture and cell quantification are described in *Supplementary Data*.

Phenotypic analysis of cultured fibrocytes

To confirm the fibrocyte phenotype of cultured cells, dual immunofluorescent labeling was performed after 9 days of culture using anti-vimentin antibody combined with antibodies against CD45, MHC class II or α-SMA. Stainings were performed as described for SMCs in *Supplementary Data*. Primary antibodies were detected using Alexa Fluor488- and HRP-conjugated secundary antibodies. HRP-activity was visualized using the TSA™ Fluorescence System (PerkinElmer LAS). Technical details of the primary and secondary antibodies used are described in *Supplementary Data*.

Since fibrocytes express active MMP-9 (gelatinase B) we performed *in situ* zymography on cultured fibrocytes. Fibrocytes were cultured for 9 days as described above. Gelatinase-activity was analyzed using the EnzCheck® Gelatinase/Collagenase Assay Kit (Invitrogen-Molecular Probes). Technical details of the *in situ* zymography procedure are described in *Supplementary Data*.

Statistical analysis

All data are expressed as mean ± SEM. For statistical analysis GraphPad Prism 4 (GraphPad Software) was used. For comparison of two group means the unpaired Student`s *t*-test was used. $p<0.05$ was considered statistically significant.

Experimental results

BN recipients are high TV-responders

Four weeks after transplantation, in BN-to-Lew allografts only weak TV developed although an increase with time was observed reaching maximal TV 24 wks after transplantation (Figure 1A, left panel & Figure 1B, white bars). Lew-to-BN allografts displayed maximal TV already after 4 wks (Figure 1A, right panel) which persisted until 8 wks after transplantation (Figure 1B, black bars). BN hosts showed significantly more pronounced TV compared with Lew hosts at 4 and 8 wks after transplantation (Figure 1B). A non-significant reduction in TV in the Lew-to-BN allografts at 12 and 24 wks was observed ($p=0.46$ *vs.* Lew-to-BN 8 wks). In BN isografts TV was hardly detected 12 wks after transplantation (Figure 1B, grey bar).

High TV-responder status is associated with BN non-MHC

BN and Lew allografts were transplanted in MHC-congenic Lew.1N and BN.1L hosts respectively to determine whether the high TV-responder status of BN recipients is associated with recipient MHC- or non-MHC-encoded determinants. Lew-to-Lew.1N allografts developed significantly less TV compared with Lew-to-BN allografts at both 4 and 8 wks after transplantation (Figure 1C). These data suggest that recipient BN non-MHC-encoded determinants predispose for accelerated development of TV rather than BN MHC. This was strengthened by the observation that in BN-to-BN.1L allografts TV tended to be more severe compared with Lew-to-Lew.1N allografts 4 wks after transplantation (Figure 1D, p=0.103). Since grafts were transplanted only in an MHC, but not non-MHC disparate combination, also DA allografts were transplanted in BN.1L and Lew.1N recipients (*i.e.* an MHC and non-MHC disparate combination). BN.1L recipients developed significantly

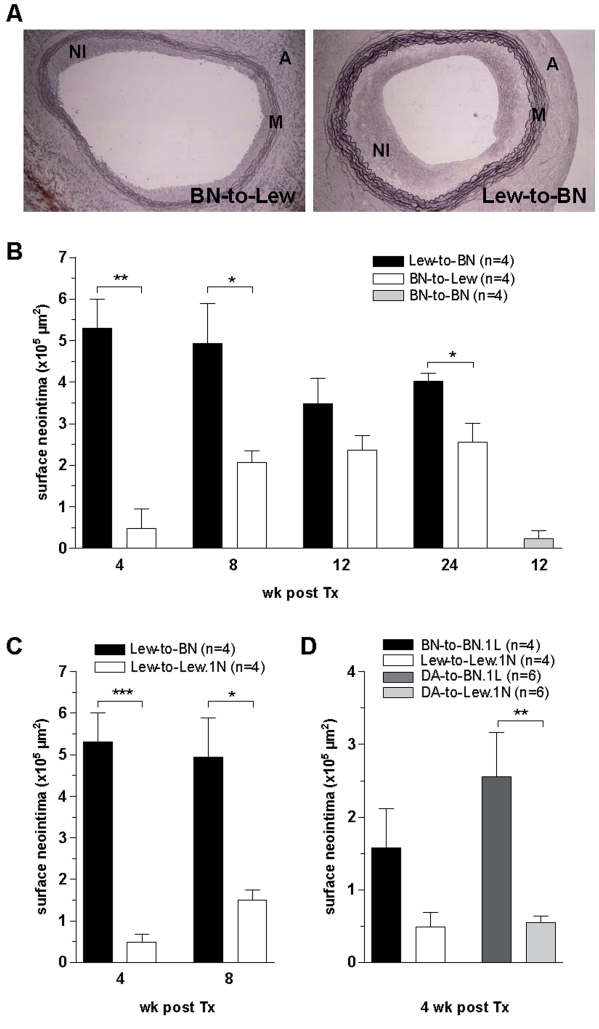
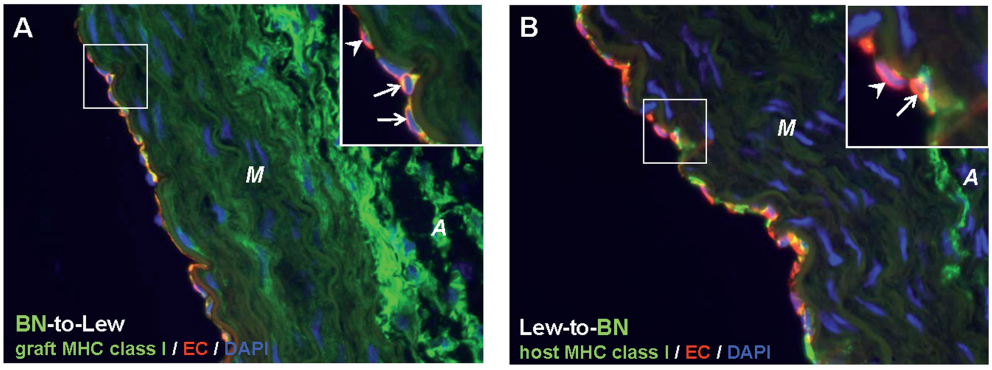


Figure 1. Kinetics and severity of the development of TV after allogeneic aorta transplantation in the BN-to-Lew, Lew-to-BN, Lew-to-Lew.1N, BN-to-BN.1L, DA-to-BN.1L and DA-to-Lew.1N rat strain combinations. **(A)** Representative photomicrographs of the severity of TV in BN-to-Lew (left panel) and Lew-to-BN allografts (right panel) 4 wks after transplantation. Lawson staining, magnification 50x. Abbreviations: A: adventitia, M: media, NI: neointima. **(B)** Quantification of surface neointima in BN-to-Lew and Lew-to-BN allografts explanted after 4, 8, 12 and 24 wks. BN isografts were after explanted 12 wks. **(C)** Quantification of surface neointima in Lew-to-BN (black bars; same data as shown in **B**) and Lew-to-Lew.1N (white bars) allografts after 4 and 8 wks. **(D)** Quantification of surface neointima in BN-to-BN.1L (black bar), Lew-to-Lew.1N (white bar), DA-to-BN.1L (dark grey bar) and DA-to-Lew.1N (light grey bar) allografts after 4 wks. (*p<0.05, **p<0.01 ***p<0.001).



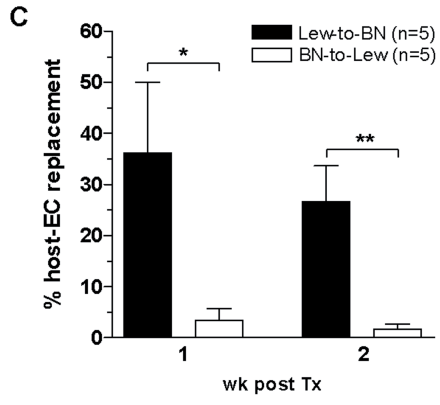


Figure 2. Host EC-chimerism in aortic allografts is increased in BN. Dual immunolabeling for HIS52$^+$ ECs (red) and OX27$^+$ BN MHC class I (green) of a Lew-to-BN **(A)** and BN-to-Lew **(B)** allograft 7 days after transplantation (magnification: 400x). Graft-derived ECs in **A** are OX27$^+$HIS52$^+$ whereas host-derived ECs are OX27$^-$HIS52$^+$. Arrowhead: host-derived OX27$^-$HIS52$^+$ EC; arrows: graft-derived OX27$^+$HIS52$^+$ ECs. Graft-derived ECs in **B** are OX27$^-$HIS52$^+$ whereas host-derived ECs are OX27$^+$HIS52$^+$. Arrowhead: graft-derived OX27$^-$HIS52$^+$ EC; arrow: host-derived OX27$^+$HIS52$^+$ EC. Insets show higher-power magnifications of the framed areas. **(C)** Quantitative analysis of the level of host EC-chimerism in Lew-to-BN and BN-to-Lew allografts. Data expressed as the percentage host-derived ECs of all detectable HIS52$^+$ ECs. (*p<0.05, **p<0.01).

more pronounced TV compared with Lew.1N recipients 4 wks after transplantation (Figure 1D). These data indicate that recipients with BN non-MHC (BN and BN.1L) are high TV-responders and recipients with Lew non-MHC (Lew and Lew.1N) are low TV-responders.

Kinetics of TV development correlates with the level of host EC-chimerism

We previously showed that in aortic allografts transplanted in BN and Lew recipients, graft endothelium is replaced with host-derived ECs (*i.e.* host EC-chimerism) at the time established TV is present.[9] Since the level of host EC-chimerism correlates with the severity of vascular damage[11], we determined the level of host EC-chimerism in aortic allografts transplanted in BN-to-Lew and Lew-to-BN allografts 1 and 2 wks after transplantation when no TV is present yet. BN-to-Lew grafts displayed near complete coverage with OX27$^+$HIS52$^+$ (graft-derived) ECs (Figure 2A). Note the expression of graft (BN) MHC class I on adventitial fibrous tissue and medial SMCs. OX27$^-$HIS52$^+$ (host-derived) ECs were sporadically detected indicating that graft endothelium is still preserved 2 wks after transplantation. In contrast, Lew-to-BN allografts contained substantial numbers of OX27$^+$HIS52$^+$ (host-derived) ECs (Figure 2B). In the BN-to-Lew combination the level of host EC-chimerism was ~2-3% at both 1 and 2 wks after transplantation whereas in

the Lew-to-BN combination the level of host EC-chimerism was significantly higher (37% and 27% at 1 and 2 wks, respectively) (Figure 2C).

TV-responder-status correlates with niSMC proliferation and ESDN expression

Since niSMCs in aortic and cardiac allografts are all host-derived[9,12] we next tested the hypothesis that the intrinsic proliferative capacity of host-derived niSMCs is associated with the kinetics and severity of TV development. niSMCs were isolated from BN-to-Lew and Lew-to-BN allografts 8-10 wks post-transplantation. Isolated neointimal cells were identified as SMCs based on morphology and growth characteristics *i.e.* a "hill and valley" morphology and expression of α-SMA (Figure 3A). Stimulation of niSMCs in the presence of 40% FBS revealed that Lew-to-BN niSMCs have a 3.0-fold increased proliferative response compared with BN-to-Lew niSMCs (Figure 3B). In contrast, Lew mSMCs showed a 2.6-fold increased proliferative response compared with BN mSMCs (Figure 3C). To get more insight into the molecular mechanism underlying the observed strain-dependent differences in niSMC proliferative capacity, expression levels of PDGF-BB and its receptors PDGFRα and PDGFRβ were determined in niSMCs using real-time RT-PCR analysis. No differences between Lew-to-BN and BN-to-Lew niSMCs were observed (not shown). However, Lew-to-BN niSMCs displayed 6-fold increased ESDN mRNA expression levels compared with BN-to-Lew niSMCs (Figure 3D).

To analyze niSMC proliferation *in situ*, immunohistochemistry for Ki67 was performed on BN-to-Lew and Lew-to-BN allografts that were harvested 4 weeks after transplantation.

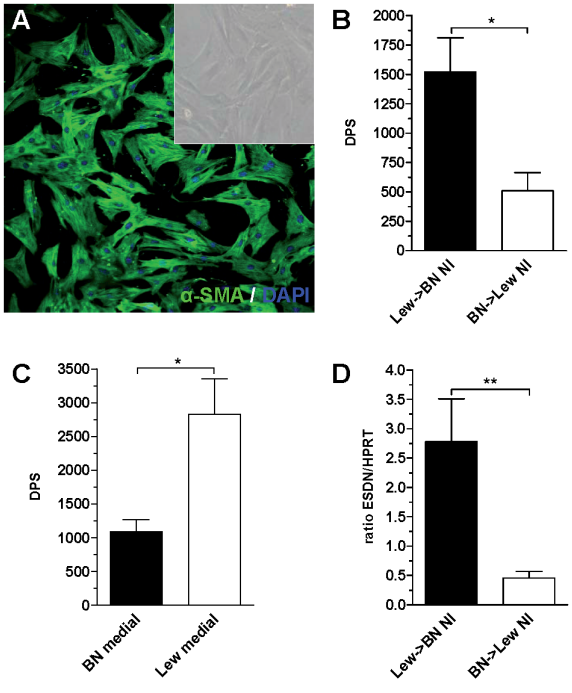


Figure 3. Intrinsic niSMC proliferative capacity correlates with TV-responder-status and ESDN mRNA expression. (A) Photomicrograph of cultured niSMCs expressing α-SMA (magnification: 200x). Inset: bright-field microscopy of cultured niSMCs (magnification: 200x). **(B)** Cultured Lew-to-BN niSMCs (black bar, N=19) are hyperresponsive compared with BN-to-Lew niSMCs (white bar, N=8). Data expressed as disintegrations per second (dps). **(C)** Cultured Lew mSMCs (white bar, N=7) are hyperresponsive compared with BN mSMCs (black bar, N=5). **(D)** ESDN mRNA expression levels were analyzed in Lew-to-BN (N=6) and BN-to-Lew (N=4) niSMCs by real-time RT-PCR analysis and expressed as the ESDN/HPRT ratio. N indicates the number of independent *in vitro* experiments performed using different passages of 3 different cell isolates of each indicated source. (*p<0.05, **p<0.01)

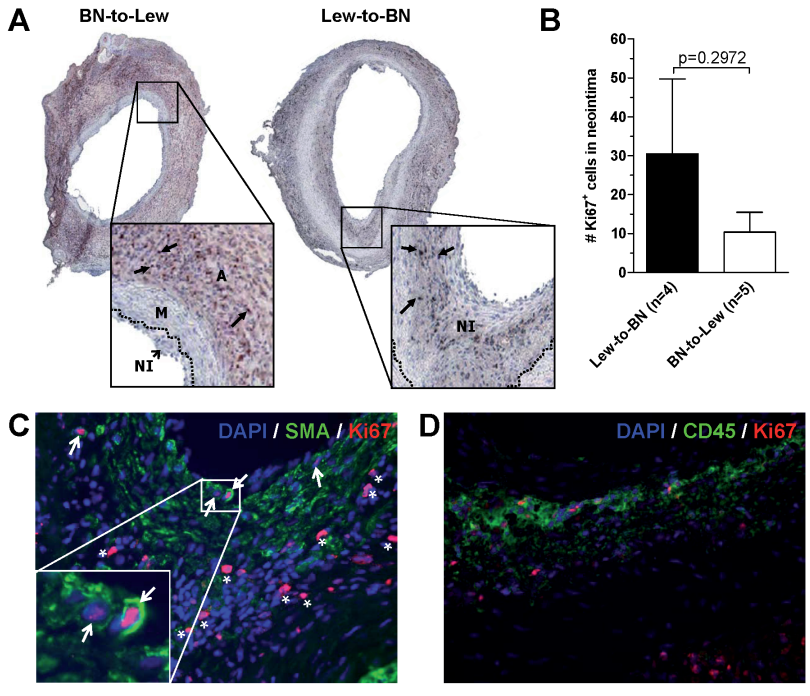


Figure 4. Lew-to-BN allografts tend to contain increased numbers of Ki67⁺ proliferating neointimal cells compared with BN-to-Lew allografts. (A) Ki67 staining on a BN-to-Lew (left) and Lew-to-BN (right) allograft. Arrows indicate Ki67⁺ nuclei. Dotted line in insets represents the internal elastic lamina. Abbreviations: A: adventitia; M: media; NI: neointima. **(B)** Quantitation of the total numbers of Ki67⁺ neointimal cells/cross-section. (Data presented as mean±SEM). **(C)** Immunofluorescent double labeling for SMA/Ki67 on the Lew-to-BN allograft shown in panel A. Arrows: Ki67⁺/SMA⁺ cells; asterisks: Ki67⁺/SMA⁻ cells. **(D)** Immunofluorescent double labeling for CD45/Ki67 on the Lew-to-BN allograft shown in panel A.

Results obtained suggest that the Lew-to-BN neointima contains increased numbers of Ki67⁺ proliferating cells (Figures 4A & B). Although neointimal lesions clearly contain proliferating cells, most proliferating cells were detected in the adventitia (Figure 4A). Quantitation of the numbers of Ki67⁺ neointimal cells revealed a tendency towards increased numbers of proliferating neointimal cells in the Lew-to-BN combination compared with the BN-to-Lew combination however without reaching the level of statistical significance (p=0.2972) (Figure 4B). Since neointimal lesions also contain infiltrating inflammatory cells immunofluorescence double-labeling for Ki67/SMA and Ki67/CD45 was performed. Proliferating Ki67⁺/SMA⁺ niSMCs were clearly present in the neointima albeit at low numbers (Figure 4C, arrows). Most proliferating cells were however SMA⁻ and were localized in area's containing large numbers of CD45⁺ cells (Figure 4D). These data suggest that neointimal lesions in Lew-to-BN allografts indeed contain more Ki67⁺ proliferating cells (both SMA⁺ niSMCs and CD45⁺ cells) compared with BN-to-Lew allografts. This may reflect a higher proliferative capacity of niSMCs which is in line with the *in vitro* data described above.

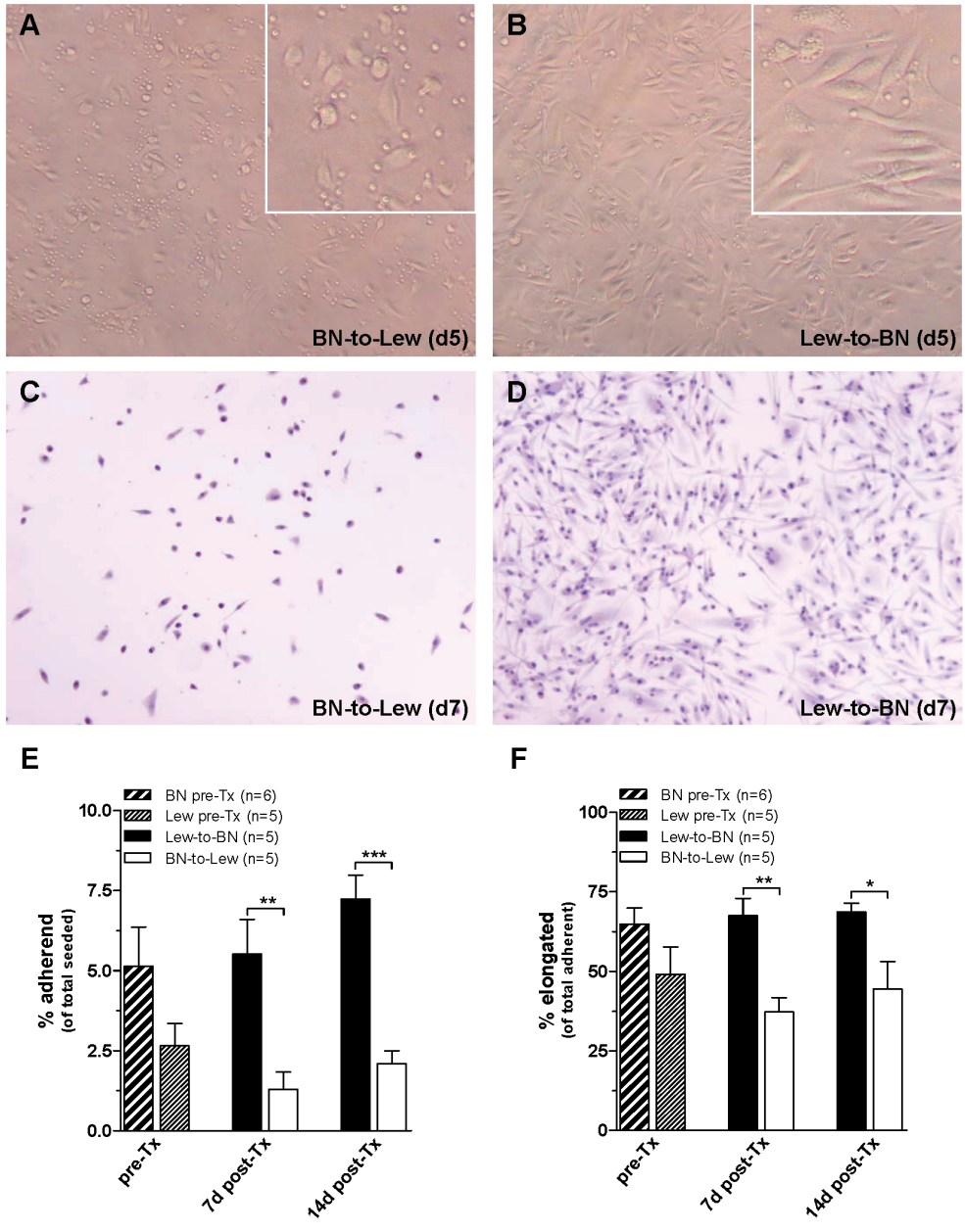


Figure 5. BN rats have an increased fibrocyte frequency compared with Lew rats. **(A)** Lew recipient PBMCs and **(B)** BN recipient PBMCs after 5 days of culture. (magnification: 200x). Insets: high-power magnifications. **(C)** Photomicrograph of hematoxylin-stained Lew recipient PBMCs and **(D)** BN recipient PBMCs after 7 days of culture. (magnification: 100x). The 7 days cultures were used for quantitative analyses. **(E)** The percentages of adherent and **(F)** elongated cells were increased in BN-derived cell cultures (black bars) compared with Lew-derived cell cultures (white bars) at both 1 wk and 2 wks after transplantation. (*p<0.05, **p<0.01, ***p<0.001).

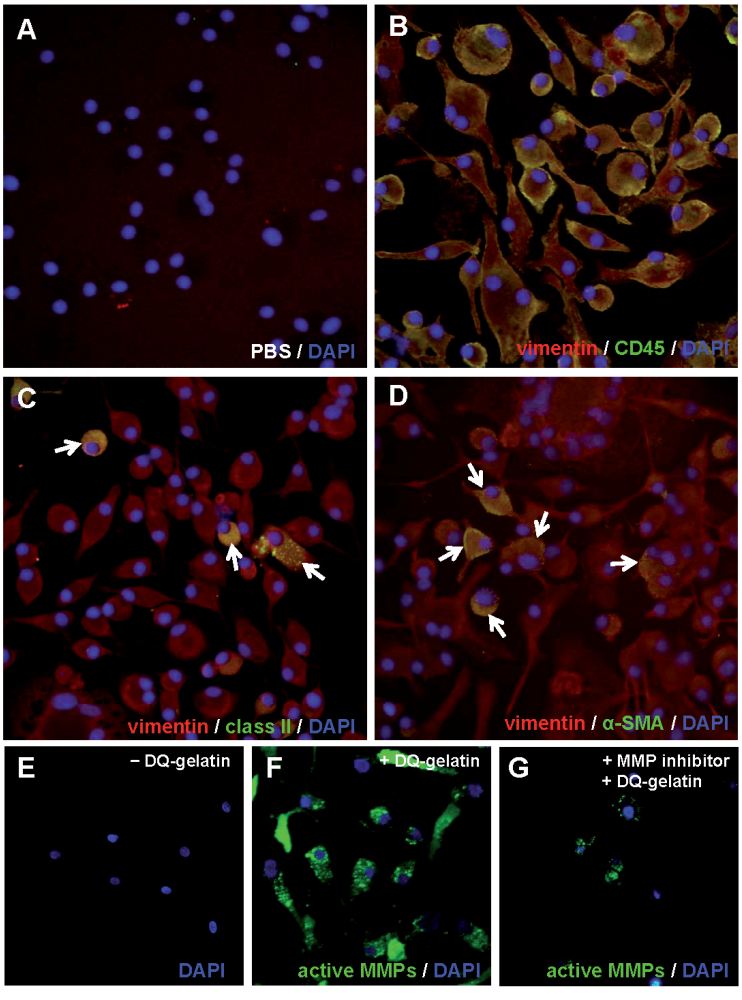


Figure 6. *In vitro* cultured fibrocytes express vimentin and CD45 and express active gelatinases. After 9 days of culture, BN fibrocytes were stained for **(B)** vimentin (red) and CD45 (green), **(C)** vimentin (red) and MHC class II (green), and **(D)** vimentin (red) and α-SMA (green). Primary incubation with PBS (no primary antibodies added) **(A)** served as a negative control. Nuclei were counterstained with DAPI. (magnification: 630x). Arrows in C and D indicate vimentin^{+}MHC class II^{+} and vimetin^{+}α-SMA^{+} double-labeled fibrocytes, respectively. Additionally, *in situ* zymography was performed after 9 days of culture. BN fibrocytes were incubated in the absence **(E)** or presence **(F)** of DQ^{TM}-gelatin (50 μg/ml) during the final 3 hrs of culture. **(G)** As a specificity control, cells were pre-incubated for 2 hrs with the general MMP inhibitor 1,10-phenanthroline (2mM). Nuclei were counterstained with DAPI. (magnification: 630x)

TV-responder-status correlates with increased circulating fibrocyte frequency

The precise anatomical origin of host-derived niSMCs in rat aorta allografts is as yet unknown but they may originate from a pool of circulating fibrocytes. Fibrocytes are mesenchymal progenitor cells exhibiting morphological characteristics of hematopoietic stem cells, monocytes and fibroblasts.[13] The frequency of circulating fibrocytes was determined *in vitro* in the BN-to-Lew and Lew-to-BN combinations before, and 1 & 2 wks after transplantation.

One day after cell seeding, no morphological differences were observed between BN- and Lew-derived PBMCs and cells appeared as a homogeneous population of small rounded non-elongated cells. However, culture of 5 days revealed a heterogeneous population of adherent cells consisting of rounded and spindle-shaped elongated (myo)fibroblast-like cells. In Lew PBMC cultures relatively few adherent elongated, spindle-shaped cells were detected (Figure 5A [5 days] & C [7 days]). In contrast, BN PBMC cultures contained large numbers of adherent elongated, spindle-shaped cells (Figure 5B [5 days] & D [7 days]). Cultures of BN PBMCs isolated from allografted recipients contained higher numbers of adherent cells (Figure 5E, black bars) as well as elongated spindle-shaped myofibroblast-like cells (Figure 5F, black bars) compared with Lew PBMCs isolated from allografted recipients (white bars). A similar pattern was observed when analyzing PBMCs isolated from non-transplanted naïve BN and Lew rats (hatched bars), however without reaching the level of statistical significance.

In vitro cultured fibrocytes express vimentin, CD45 and gelatinase activity

To confirm the fibrocyte-phenotype of adherent elongated spindle-shaped cells in the BN PBMC cultures dual immunofluorescent labeling for vimentin/CD45, vimentin/MHC class II or vimentin/α-SMA was performed. As shown in Figure 6, virtually all BN PBMC-derived adherent elongated cells co-expressed vimentin and CD45 (Figure 6B), whereas co-expression of vimentin and MHC class II (Figure 6C) or α-SMA$^+$ (Figure 6D) was detected in the minority of cells. Since human fibrocytes were previously shown to express latent and active MMP-9 (gelatinaseB) but not MMP-2 (gelatinaseA)[14] we determined gelatinase-activity in our culture system. BN PBMC-derived adherent elongated cells expressed active gelatinases as indicated by degradation of DQ^{TM}-gelatin using *in situ* zymography (Figure 6F). In the absence of DQ^{TM}-gelatin no fluorescence was detected (Figure 6E), whereas pre-incubation with the general inhibitor of MMPs 1,10-phenanthroline reduced gelatinase activity (Figure 6G).

Discussion

Strain-dependent heterogeneity in various models of vascular remodeling except TV has been demonstrated in mice and rats[15-17] although underlying cellular mechanisms are still unknown. We studied potential cellular mechanisms associated with TV susceptibility using a well-established experimental aortic transplant model in rats.[9] This model allows isolation and functional characterization of niSMCs *ex vivo* which is not feasible after experimental solid organ transplantation. The main findings of this study are: 1) BN rats are high TV-responders whereas Lew rats are low TV-responders. The high BN TV-responder-status is associated with 2) presence of BN non-MHC-encoded determinants, 3) increased levels of intragraft host EC-chimerism indicative of EC damage, and 4) an increased endogenous pro-fibrotic state as indicated by increased intrinsic niSMC proliferative capacity and ESDN expression levels, and increased frequency of putative niSMC ancestral cells.

In this study, two rat strains were identified that represent two extreme phenotypes with respect to the kinetics of TV development. In addition to the BN-to-Lew and Lew-to-BN

combinations, severity of TV development was also analyzed in the PVG-to-AO and DA-to-WF strain combinations which revealed intermediate development of TV 4 weeks after transplantation (not shown). Collectively, these results indicate that BN rats can be defined as high TV-responders and Lew rats as low TV-responders. This responder-status appears not to be specific for TV since in a restenosis model, BN rats developed more pronounced restenosis than Lew rats.[18] More in-depth linkage analysis of restenosis in BN and SHR rats revealed several quantitative trait loci intervals encoding, among others, MMP-9 and Cadherin-22.[19] Transplantations performed in MHC-congenic Lew.1N and BN.1L rats furthermore revealed that the high BN TV-responder-status is determined by non-MHC-encoded, but not MHC-encoded, determinants. The immunologic responder-status in rats (Th1 *vs.* Th2) is not genetically linked to the MHC and therefore independent of the MHC haplotype. Despite the Th2-type responder-status and high TV responsiveness of BN rats, Th2-skewed F344 rats are low TV-responders resembling Th1-skewed Lew rats[20,21] which favors against a Th2-type immunologic responder-status as a predominant predisposing factor for TV development.

EC injury is considered as the inciting event in TV development[22] and maintaining endothelial integrity supposedly leads to reduced TV. We analyzed the level of host EC-chimerism as a marker for EC injury[11] in the early post-transplant period. Compared with Lew, BN recipients displayed increased levels of host EC-chimerism suggesting more severe graft EC damage in BN recipients. In line with this, Lew-to-BN allografts were characterized by earlier and more aggressive acute vascular rejection compared with BN-to-Lew allografts.[23,24] In addition to this aggravated damage-inducing rejection response in BN recipients, also the vulnerability of graft ECs for damage might influence the final outcome of TV development. In a myocardial ischemia model, BN hearts were shown to be more resistant to ischemia[25] which could imply that also BN aortic grafts are less susceptible to ischemia/reperfusion injury than Lew grafts. Less EC damage in the peri-operative period might then translate into reduced TV development.

Since we previously showed host-origin of niSMCs in aortic and cardiac allografts[9,12], we here analyzed whether the TV-responder-status is linked to the intrinsic niSMC proliferative capacity and availability of host-derived fibrocytes. Fibrocytes are considered as a population of circulating putative niSMC ancestral cells. niSMCs isolated from Lew-to-BN grafts (niSMCs of BN origin) showed increased proliferative responses compared with BN-to-Lew niSMCs (niSMCs of Lew origin). In contrast to niSMCs, mSMCs from Lew and BN rats responded in the opposite manner. Lew mSMCs showed significantly increased proliferation rates compared with BN mSMCs. These differences between niSMC and mSMC proliferative capacity strengthen our previous observation that niSMCs are most likely derived from a pool of circulating progenitor cells and not mSMCs[9,26], and differ, at least at the proliferative level, from mSMCs.[27-29]

Also *in situ*, neointimal lesions in Lew-to-BN allografts appeared to contain more $Ki67^+$ proliferating cells compared with BN-to-Lew allografts. This may reflect a higher proliferative capacity of niSMCs which would be in line with our *in vitro* data. The proliferating cell population consisted of both SMA^+ niSMCs and $CD45^+$ cells. The proliferating $CD45^+$ cells may actually represent $CD45^+$ fibrocytes but this needs further confirmation.

The difference in niSMC proliferative capacity might be genetically determined, although the responsible gene product(s) is as yet unknown. Genetic variance in the expression and regulation of growth factors involved in neointima formation and SMC proliferation (*e.g.* VEGF and PDGF) might be involved in determining the proliferative capacity of niSMCs *in vivo* in a paracrine manner. Indeed, genetic polymorphisms in the VEGF and PDGF genes have been linked to the development of TV in cardiac allografts and renal allograft rejection.[7,8,30] However, in our experimental setup niSMCs were exposed to standardized culture conditions thereby making availability of, and exposure to, growth factors unlikely to be the underlying cause of differential niSMC proliferative capacity. Alternatively, niSMC proliferative capacity might be intrinsically determined and is thereby independent of the microenvironment in which niSMCs reside. The identification of ESDN as a selective marker for niSMC proliferation in vascular remodeling including TV[31] led us to test the expression levels of ESDN in niSMCs. ESDN is induced in niSMCs upon mechanical and allo-immune-mediated vascular damage *in vivo*.[31] Although the exact function of ESDN remains unclear, proliferating niSMC clearly express ESDN in which it may function as a negative regulator of proliferation.[31,32] In line with this Guo et al. recently showed that downregulation of ESDN expression was associated with PDGF-induced PDGFRß ubiquitination. These data suggest that ESDN may function as a negative regulator of SMC proliferation by modulating PDGF signaling via regulation of PDGFRß surface levels[33]. In this respect the level of ESDN expression might be considered as a marker for SMC proliferative capacity. We confirmed expression of ESDN mRNA in *in vitro* cultured niSMCs and furthermore showed the expression level to be increased in the more highly proliferative niSMCs.

niSMCs in aortic allografts are host-derived[9] and most likely blood-borne[10] and may originate from a population of circulating smooth muscle progenitor cells. Smooth muscle progenitor cells reside in the human peripheral blood and are possibly $CD14^{+}$[34,35], but may lose myeloid markers after *in vitro* culture.[36] These monocyte-derived smooth muscle progenitor cells resemble, or may even be identical to, fibrocytes. Fibrocytes form a population of circulating mesenchymal progenitor cells exhibiting morphological characteristics of hematopoietic stem cells, monocytes and fibroblasts.[13] Fibrocytes can migrate to wound sites *in vivo*[37] and differentiate into α-SMA-expressing myofibroblasts which is promoted by TGF-ß[13]. Moreover, fibrocytes were shown to contribute to intimal hyperplasia in an ovine synthetic patch graft model.[38] We demonstrated significantly higher numbers of circulating fibrocytes in BN rats compared with Lew rats suggesting that, in addition to niSMC proliferative capacity, also the frequency of circulating putative niSMC progenitor cells determines TV-responder-status. Since our findings do not provide experimental proof that circulating fibrocytes are the ancestors of niSMCs, a paracrine modulatory role in neointima formation cannot be excluded.

Conclusions

We showed that susceptibility to develop TV is likely under genetic control affecting niSMC proliferative capacity and the putative niSMC progenitor cell compartment. Based on our results, future studies should focus on dissecting the genetic/molecular pathways that cause the observed heterogeneity within the niSMC compartment. When translating our experimental data to the human setting, unraveling the genetic basis of niSMC proliferation and fibrocyte frequency will help to identify transplant patients at risk and to reveal molecular targets that can potentially serve as drugable targets to prevent or treat TV development.

Acknowledgments

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Supplementary data

Materials and methods

Evaluation of host EC-chimerism in aortic allografts

Host EC-chimerism was determined in grafts transplanted in the Lew-to-BN and BN-to-Lew combinations explanted 1 and 2 wks after transplantation. Cryosections (5 μm) were stained using dual immunolabeling with endothelial-specific (clone HIS52/RECA-1, mIgG1)[1] and BN MHC-class I-specific (clone OX27, mIgG2a)[2] mAbs in order to determine the origin (graft *vs.* recipient) of ECs at the luminal side of the graft. One hour incubation with primary mAbs was followed by 30 min incubation with TRITC-conjugated goat anti-mIgG1 and FITC-conjugated goat anti-mIgG2a (both from Southern Biotechnology Associates) isotype-specific second-step antibodies diluted in PBS/1% BSA/1% normal rat serum. Nuclei were counterstained with DAPI and slides were coverslipped in Citifluor (AF1, Agar Scientific Ltd.). Graft ECs were analyzed using a Leica DMRXA fluorescent microscope and pictures were taken using Leica Qwin software (Leica Microsystems). Total numbers of ECs (RECA-1$^+$) and double-labeled ECs (RECA-1$^+$OX27$^+$) were counted using AnalySIS software (Soft Imaging System). Host EC-chimerism was expressed as the percentage of host-derived ECs of the total number of ECs present per cross-section. Depending on the integrity of the EC monolayer, 1 to 4 sections per graft were analyzed.

Rat medial and neointimal SMC isolation, culture and phenotyping

For niSMC isolation, abdominal aorta transplantations were performed in Lew-to-BN and BN-to-Lew combinations. Grafts were explanted 8-10 wks post-transplantation, opened longitudinally after which the endothelium was gently removed using a cotton swab. Using forceps, the neointima was then carefully dissected from the adjacent media and minced in small pieces of ~1 mm^2. For cell dispersion, neointimal tissue fragments were treated with collagenase (3.5 mg/mL) for 1 hr. After removel of collagenase, a mixture of elastase (1 mg/mL) and soybean trypsin inhibitor (0.1 mg/mL) was added and tissue fragments were incubated for another hour after which freshly prepared collagenase was added at a final concentration of 1.75 mg/ml and incubated for 1.5 hr. During the final 10 min. of incubation, DNase (final concentration 0.05 mg/mL) was added. All enzymes were obtained from Sigma Aldrich. Incubations were performed at 37°C in a rocking waterbath. For mSMC isolation, freshly collected thoracic aortas were used. Peri-aortic tissue was

removed after which the aorta was opened longitudinally and the endothelium removed. The tunica media was then separated from adjacent adventitia, cut into small fragments and enzymatically digested as described above. Both mSMCs and niSMCs were cultured in SMC growth medium (EMEM supplemented with 4 mM glutamine, 100 mM NEAA [all from Invitrogen], 10% FBS [Perbio Science] and 50 μg/mL gentamycin). Cells were identified as SMCs based on their morphology (*i.e.* typical "hill and valley" morphology) and growth characteristics as reported previously.[3] Phenotyping of isolated and cultured mSMCs and niSMCs was performed by analyzing α-SMA expression. To this end, mSMCs and niSMCs were grown on 8-well Lab-Tek chamber slides (Nalge Nunc International) in SMC growth medium until they reached 80% confluency. Subsequently, cells were growth-arrested for 24 hrs in SMC starvation medium with 0.4% FBS and then fixed in 1:1 acetone:methanol at -20°C for 20 minutes. Fixed cells were stained for α-SMA (clone 1A4, mIgG2a, Dako). One hour incubation with the primary antibody was followed by incubation with FITC-conjugated goat-anti-mIgG2a (Southern Biotechnology Associates) isotype-specific second-step antibody. Nuclei were counterstained with DAPI and slides were coverslipped in Citifluor (AF1, Agar Scientific Ltd.) Stained cells were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems).

Real-time RT-PCR analysis

mRNA expression levels of Endothelial and Smooth Muscle Cell-derived Neuropilin-like Protein (ESDN), platelet-derived growth factor (PDGF)-BB and its receptors PDGFRα and PDGFRβ were determined using real-time RT-PCR analysis. To this end, total RNA from cultured Lew-to-BN and BN-to-Lew niSMCs was isolated using TRIreagent (Sigma Aldrich Chemie B.V.) according to the manufacturer's instructions. DNase treatment (Sigma Aldrich Chemie B.V.) was performed to avoid contamination with genomic DNA. cDNA was prepared using M-MuLV Reverse Transcriptase (Fermentas). Transcript levels of ESDN, PDGF-BB, PDGFRα, PDGFRβ and the house-keeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) were quantified by real-time PCR using ABsolute™ QPCR SYBR® Green Fluorescein Mix (Westburg) on an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories B.V.). Primer sequences and product size are given in Table 1 of the *Supplementary Material*. Results were expressed as the target gene/HPRT ratio according to a mathematical method described elsewhere.[4]

Quantification of the frequency of circulating fibrocytes

Heparinized blood was collected by cardiac puncture before, and 7 and 14 days after transplantation. PBMCs were isolated by Ficoll density gradient (Histopaque 1083, Sigma Aldrich Chemie B.V.) centrifugation following the manufacturer's instructions. Isolated PBMCs were resuspended in low-glucose (1 g/L) DMEM (PAA Laboratories) (supplemented with 20% FBS and 60 μg/mL gentamycin) and plated ($0.5x10^6$ cells/well) in 8-well Lab-Tek chamber slides (Nalge Nunc International) that were pre-coated with 400 μl/well human recombinant fibronectin (15 μg/mL) (BD Biosciences). After 4 days of culture, medium was replenished and non-adherent cells were removed. After 7 days of culture, cells were washed

with PBS and then fixed in 1:1 acetone:methanol at -20°C for 12 min. Cells were stained with Mayer`s hematoxylin and coverslipped with Kaiser`s glycerol-gelatin. Histomorphometric analyses were performed using AnalySIS software (Soft Imaging System) by counting the number of spindle-shaped cells in 4 randomly selected fields (magnification 100x) per well.

Phenotypic analysis of cultured fibrocytes

To confirm the fibrocyte phenotype of cultured cells, dual immunofluorescent labeling was performed after 9 days of culture using anti-vimentin antibody (goat polyclonal IgG, sc-7557, Santa Cruz Biotechnology Inc.) combined with antibodies against CD45 (clone OX-1, mIgG1, tissue culture supernatant), MHC class II (clone OX-6, mIgG1, tissue culture supernatant) or α-SMA (clone 1A4, mIgG2a, Dako). Stainings were performed as described above for mSMCs and niSMCs. CD45/MHC class II and α-SMA primary antibodies were detected using Alexa Fluor488-conjugated goat-anti-mIgG1 and Alexa Fluor488-conjugated goat-anti-mIgG2a isotype-specific second-step antibodies (both from Invitrogen-Molecular Probes), respectively. Vimentin primary antibody was detected using HRP-conjugated rabbit-anti-goat secondary antibody (Dako). HRP-activity was visualized using the TSA™ Fluorescence System (PerkinElmer LAS, Inc., Boston, MA, USA) with Cy5 as tyramide label.

In situ zymography on cultured fibrocytes

Since fibrocytes express active MMP-9 (gelatinase B)[5,6] we performed *in situ* zymography on cultured fibrocytes. Fibrocytes were cultured for 9 days as described above. DQ™-gelatin (EnzCheck® Gelatinase/Collagenase Assay Kit, Invitrogen-Molecular Probes) was then added to the culture medium at a final concentration of 50 μg/ml and incubated for 3 hrs after which the cells were washed twice with PBS. Nuclei were counterstained with DAPI and slides were coverslipped in Citifluor (AF1, Agar Scientific Ltd.). Stained cells were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems). To control for specificity, cells were either incubated in the absence of DQ™-gelatin or pre-incubated with 2 mM 1,10-phenanthroline, a general inhibitor of MMPs, for 2 hrs.

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Table 1. Primer sequences used for real-time RT-PCR analysis

Gene	Accession number		Primer sequence	Product size
ESDN	NM130419	FW	5`-GTGGAATCCATGCTTCTGGT-3`	132 bp
		REV	5`-AGCCAGCTGGGCAGTACTTA-3`	
PDGF-BB	XM001075973	FW	5'-GAAGCCAGTCTTCAAGAAGGCCAC-3'	148 bp
		REV	5'-AACGGTCACCCGAGTTTGAGGTGT-3'	
PDGFRα	NM012802.1	FW	5'-GCCAGTTACAGGAAGCTGTC-3'	168 bp
		REV	5'-AGAGGAACAGACACAGCTCG-3'	
PDGFRβ	NM031525.1	FW	5'-TGCTCACCACCTCATATTCC-3'	158 bp
		REV	5'-TGCCTCAGCCAAATGTCACC-3'	
HPRT	NM012583	FW	5`-GCGAAAGTGGAAAAGCCAAGT-3`	76 bp
		REV	5`-GCCACATCAACAGGACTCTTGTAG-3`	

Abbreviations: bp: base pairs; ESDN: endothelial and smooth muscle cell-derived neuropilin-like protein; FW: forward; HPRT: hypoxanthine guanine phosphoribosyl transferase; PDGF-BB: platelet-derived growth factor-BB; PDGFRα: platelet-derived growth factor receptor alpha; PDGFRβ: platelet-derived growth factor receptor beta; REV: reverse

4

Rosiglitazone attenuates transplant arteriosclerosis after allogeneic aorta transplantation in rats

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Abstract

Background. Transplant arteriosclerosis is a leading cause of chronic transplant dysfunction and is characterized by occlusive neointima formation in intragraft arteries. Development of transplant arteriosclerosis is refractory to conventional immunosuppressive drugs and adequate therapy is not available. In this study we determined the efficacy of the synthetic peroxisome proliferator-activated receptor-γ (PPARγ) agonist rosiglitazone to attenuate the development of transplant arteriosclerosis in rat aortic allografts.

Methods. Lewis aortic allografts were transplanted into Brown Norway recipient rats. Recipients rats received either ~5 mg rosiglitazone/day (starting 1 wk before transplantation until the end of the experiment) or were left untreated. Transplant arteriosclerosis was quantified using morphometric analysis. Alloreactivity was measured *in vitro* using mixed lymphocyte reactions. Regulatory T cell frequency and function were analyzed using flowcytometry and *in vitro* suppression assays, respectively. Intragraft gene expression was analyzed using real time PCR. Finally, medial and neointimal vascular smooth muscle cell proliferation was analyzed *in vitro*.

Results. Rosiglitazone significantly reduced TA development 8 wks after transplantation ($p<0.01$ *vs.* non-treated). Rosiglitazone reduced T cell alloreactivity which was not mediated through modulation of $CD4^+CD25^+FoxP3^+$ regulatory T cells. Reduced development of transplant arteriosclerosis coincided with reduced intragraft expression of SDF-1α and PDGFRβ. Finally, rosiglitazone reduced growth-factor-driven proliferation of both medial and neointimal vascular smooth muscle cells *in vitro*, which was not mediated through PPARγ.

Conclusion. PPARγ agonists may offer a new therapeutic strategy in clinical transplantation to attenuate the development of transplant arteriosclerosis and thereby chronic transplant dysfunction.

Abbreviations

CTD chronic transplant dysfunction
PPAR-γ peroxisome proliferator-activated receptor-γ
RSG rosiglitazone
TA transplant arteriosclerosis
VSMC vascular smooth muscle cell

Introduction

Advances in graft procurement, preservation, matching and immunosuppression contributed to the outstanding short-term (<1 year) patient and graft survival after solid organ transplantation as achieved today.[1] However, these advances have not resulted in prevention of chronic transplant dysfunction (CTD) evolving long-term after transplantation (>5 years). CTD is the major cause of premature graft loss after the first postoperative year.[2] Therefore, solid organ transplantation (including heart, kidney and lung) has still not achieved its goals as a long-term treatment for patients with end-stage organ failure. CTD is the outcome of persisting inflammation-driven injury to the graft, which cannot be adequately controlled by currently used anti-rejection therapies. Common histopathological feature of CTD is transplant arteriosclerosis (TA), *i.e.* occlusive neointima formation consisting of predominantly smooth muscle α-actin expressing vascular smooth muscle cells (VSMC's).[2,3] Occlusive neointima formation supposedly leads to downstream ischemic tissue damage and disruptive fibrosis thereby making TA the putative cause of chronic transplant dysfunction.[3] Pursuing the development of new therapies aiming at attenuating TA development is therefore warranted.

Thiazolidinediones (*i.e.* rosiglitazone and pioglitazone) are synthetic agonists for peroxisome proliferator-activated receptor-γ (PPARγ). To date, thiazolidinediones are solely prescribed to Type 2 diabetics to improve insulin-resistance.[4,5] Although PPARγ is predominantly expressed in adipose tissue also vascular cells like endothelium and VSMC's are reported to express PPARγ.[5] PPARγ ligation by thiazolidinediones on vascular cells has multiple effects including enhanced nitric oxide release from endothelial cells, inhibited leukocyte-endothelial cell interactions and inhibited VSMC proliferation and migration.[6-8] In addition to their effects on adipocytes and vascular cells, thiazolidinediones are anti-inflammatory through generalized repression of transcription of inflammatory genes in leukocytes.[7,8]

Since development of TA in solid allografts is supposedly the result of inflammation-driven injury to the graft endothelium, followed by subsequent VSMC recruitment and proliferation and extracellular matrix deposition, PPARγ agonists are potential drugs to attenuate TA. Kosuge *et al.* indeed recently reported that pioglitazone is able to reduce both acute rejection and development of TA in mouse cardiac allografts.[9] In the present study we investigated the efficacy of the PPARγ agonist rosiglitazone in attenuating TA development in rat aortic allografts. In this study we show that treatment with rosiglitazone is highly effective in reducing the development of TA in non-immunosuppressed aortic allograft recipients. Mechanisms involved include reduction of the alloreactive response (immunosuppressive effect) as well as direct effects on VSMC recruitment and proliferation.

Materials and methods

Rats

Specified pathogen free male Lewis (Lew, RT-1^{l}), Brown Norway (BN, RT-1^{n}) and Wistar Furth (WF, RT-1^{u}) were obtained from Harlan (Horst, The Netherlands). Rats were between 8-10 weeks of age and were housed and treated in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and institutional guidelines.

Aorta transplantation

Under anesthesia (2% halothane, 0.4 L/min O_2 and 0.4 L/min N_2O) Lew aortic allografts were transplanted to BN recipients as described previously.[10] Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor, perfused with saline and subsequently orthotopically transplanted into the recipient via end-to-end anastomosis with total cold and warm ischemic time consistently less than 25 minutes. Recipient rats did not receive anti-rejection therapy after transplantation.

Rosiglitazone treatment

Recipient rats were maintained on standard rat chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) formulated with or without rosiglitazone (RSG) (Avandia, GlaxoSmithKline, Zeist, The Netherlands). Rosiglitazone was admixed in the chow to a final concentration of 0.026% wt/wt. Based on a measured average food intake of ~16 g/day/rat, the average rosiglitazone intake was ~4.2 mg/day/rat. Food intake between rosiglitazone-treated and non-treated rats did not differ throughout the duration of the experiment. RSG-treatment started 1 wk before transplantation and was continued throughout the experiment (8 wks). To determine actual rosiglitazone uptake from the diet, plasma levels were determined 8 wks post-transplantation by high-pressure liquid chromatography with tandem mass spectrometry using purified rosiglitazone maleate for calibration.

Measurement of neointimal area of aortic allografts

Grafts were harvested and transversally cut in the middle of the graft. The proximal part of the two segments was then embedded in paraffin facing the middle part upwards. Cross-sections (5 μm) were cut starting from the middle part of the graft and stained with Verhoeff's elastin. Surface neointima was quantified in 6 sections from each graft: 3 sections cut at the middle of the graft and 3 sections cut at least 100 μm further into the graft. Surface neointima (expressed in μm^2) was then determined in the six sections of each aortic allograft by subtracting lumen area from internal elastic lamina area. No differences were observed between the two areas from which the sections were obtained. The mean surface neointima in each graft was then calculated. All grafts were processed in exactly the same manner as described above. Morphometric analyses were performed using AnalySIS software (Soft Imaging System GmbH, Germany) and surface neointima was determined by subtracting lumen area from internal elastic lamina area. The mean surface neointima (expressed in μm^2) in each graft was then calculated.

Determination of endothelial cell replacement in aortic allografts

To determine the origin (graft *vs.* recipient) of neointimal endothelial cells, immunofluorescent double staining for recipient MHC class I and endothelium was performed on 5 µm cryosections. Primary monoclonal antibodies OX27 (BN MHC class I)[11] and RECA-1 (endothelium)[12] were detected using Alexa488-conjugated (Molecular Probes, Invitrogen, Breda, The Netherlands) and TRITC-conjugated (SouthernBiotech, Birmingham, AL, USA) isotype-specific second-step antibodies. Nuclei were counterstained with DAPI and sections were coverslipped in Citifluor. Sections were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems Nederland B.V., Rijswijk, The Netherlands).

Mixed lymphocyte reaction

To analyze the effect of rosiglitazone on T cell alloreactivity two different experimental setups were used as described in detail in *Supplementary Data*. In the first setup (MLR1), alloreactive T cell responses of rats that were RSG-treated *in vivo* (or left untreated) were measured in an *in vitro* MLR. In the second setup, MLR's were performed with splenocytes obtained from naive rats (no RSG treatment, no transplantation) and RSG was added to the cultures (MLR2). Proliferation was assessed by liquid scintillation and results were expressed as disintegrations per second (dps) (MLR1) or as percentage inhibition of proliferation relative to the vehicle control (MLR2).

Flowcytometric analysis

To determine the frequency and absolute numbers of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) in the peripheral blood of transplanted RSG-treated and non-treated rats, flowcytometric analyses were performed on peripheral blood mononuclear cells (PBMNC`s) (see *Supplementary Data*).

Isolation of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cells for *in vitro* proliferation assays were obtained from pooled mesenteric lymph node (MLN) and cervical lymph node (CLN) single-cell suspensions from transplanted RSG-treated or non-treated BN rats (6 wks after transplantation) as described in *Supplementary Data*.

Regulatory T cell suppression assays

To analyze Treg suppressor function *in vitro*, sorted $CD4^+CD25^-$ responder T cells (pooled cells isolated from four allografted, non-rosiglitazone treated BN rats) were cocultured in triplicate in 96-well plates with autologous irradiated antigen presenting cells (APCs), ConcanavalinA (10 µg/ml), and different amounts of purified $CD4^+CD25^+$ Tregs (isolated from either allografted, non-rosiglitazone treated or allografted, rosiglitazone-treated BN rats) (see *Supplementary Data*).

Enzyme-linked immunoassay

Production of IFN-γ was measured in supernatants collected from allostimulated naive splenocytes treated with rosiglitazone *in vitro* (MLR2 as described above). Supernatants were stored at −20°C until enzyme-linked immunoassay (ELISA) was performed. The concentrations of IFN-γ were detected using the OptEIA^{tm} Rat IFN-γ ELISA kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer`s instructions.

Real-time RT-PCR analysis

To investigate possible target genes involved in rosiglitazone-induced attenuated neointima formation, intragraft mRNA expression levels were determined. Grafts were removed 6 wks after transplantation, snap-frozen in liquid nitrogen and homogenized in TRIreagent (Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands) using a Pro200 homogenizer (Pro Scientific Inc., Oxford, CT, USA). To investigate PPARγ expression levels in cultured medial and neointimal VSMC's, cells were lysed in TRIreagent. Total RNA from aortic allografts and cultured VSMC's was isolated according to the manufacturer's protocol. DNase treatment (Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands) was performed to avoid contamination with genomic DNA. cDNA was prepared using M-MuLV Reverse Transcriptase (Fermentas, St Leon-Rot, Germany).

Transcript levels of the fatty acid translocase CD36, stromal-derived factor-1α (SDF-1α), platelet-derived growth factor receptor-β (PDGFRβ), peroxisome proliferator-activated receptor-γ (PPARγ) and hypoxanthine guanine phosphoribosyl transferase (HPRT; house-keeping gene) were quantified by real-time PCR using ABsolute™ QPCR SYBR® Green Fluorescein Mix (Westburg, Leusden, The Netherlands) on an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Results were expressed as a relative ratio to the house-keeping gene HPRT according to a mathematical method as described.[13] Primer sequences, PCR product sizes and annealing temperatures are listed in Table 1 in *Supplementary Data*.

Rat medial and neointimal VSMC proliferation

Medial VSMC`s from thoracic aorta of Lew and BN rats were isolated using enzymatic digestion as described.[14] For neointimal VSMC isolation, abdominal aorta transplantation was performed in the Lew-to-BN strain combination. Grafts were explanted 10 wks post-transplantation and neointimal cells isolated, cultured and stimulated *in vitro* with 25 ng/ml PDGF-BB (R&D Systems, Europe Ltd., Abingdon, UK) in the presence of 100 μM rosiglitazone or 1% EtOH (vehicle control). Rosiglitazone was added 30 minutes before adding PDGF-BB. To analyze whether VSMC proliferation is modulated by rosiglitazone through PPARγ, cells were pretreated 30 minutes with 5 μM GW9662, a PPARγ antagonist (Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands) or vehicle (0.05% DMSO). For details, see *Supplementary Data*.

Statistics

Data are presented as the mean ± SD or mean ± SEM as indicated. Comparisons of two means used the unpaired *t* test. Differences between groups with n<4 were analyzed using a two-tailed Mann Whitney *U* test. For evaluation of proliferation data in relation with their vehicle controls as wells as for IFN-γ production in MLR supernatants, paired *t* tests were performed. Values of p≤0.05 were considered statistically significant.

Results

Rosiglitazone attenuates transplant arteriosclerosis

RSG admixed in the diet entered the circulation and was biologically active as indicated by increased intragraft CD36 expression (data available in figure 1, *Supplementary Data*). No

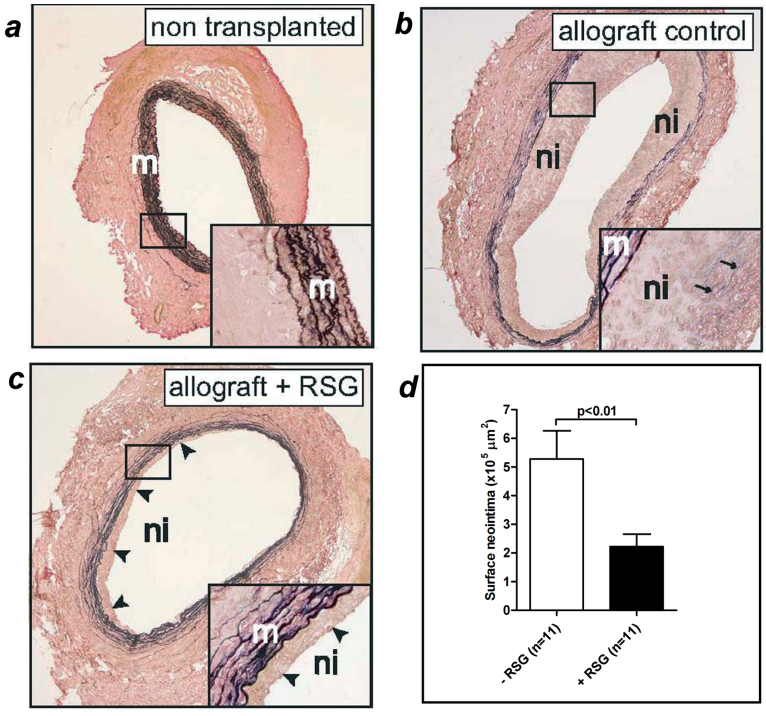

Figure 1. Rosiglitazone significantly reduces neointima formation in aortic allografts. BN recipient rats in the treatment group were fed diet containing 0.026% wt/wt rosiglitazone. Rats in the non-treated control group were fed diet with the same formula except RSG. (**a**) Morphology of a non-transplanted aorta with a well preserved media and without adventitial inflammation (magnification x40). Inset shows a high-power magnification (x400) of the adventitia/media. (**b**) Control group (no rosiglitazone treatment) (magnification x40). Neointima in the control group is characterized by elastin deposition (inset, ↑) which was absent in the treated group. (**c**) RSG treatment resulted in reduced neointima formation (indicated by arrowheads) compared with the non-treated control group. Insets in *b* & *c* show high-power magnifications (x400) of the media/neointima. (**d**) Rosiglitazone treatment significantly reduced neointima formation as quantified by computerized morphometric analysis. Data are expressed as mean ± SEM, p<0.01, unpaired *t* test. Staining performed on the sections shown in *a-c*: Verhoeff's elastin staining. Abbreviations: m: media; ni: neointima.

side-effects were recorded in the RSG-treated rats. To investigate the effect of rosiglitazone on TA development, fully MHC-incompatible Lew aortic allografts were transplanted into BN recipient rats. Representative microphotographs of vascular wall morphology are shown in Figure 1a-c. Aortic allografts from non-treated rats presented with severe neointima formation. RSG treatment resulted in a significant reduction in the surface neointima present in the allografts (57% reduction, $p<0.01$ *vs.* non-treated controls) (Figure 1d).

Graft endothelial cells after rosiglitazone treatment are of host-origin

Using recipient-specific immunofluorescent staining, we determined the origin (graft *vs.* recipient) of the endothelial cells (EC`s) of the aortic allografts. Double immunolabeling for EC`s and recipient MHC class I revealed host-origin (positive staining for both the EC

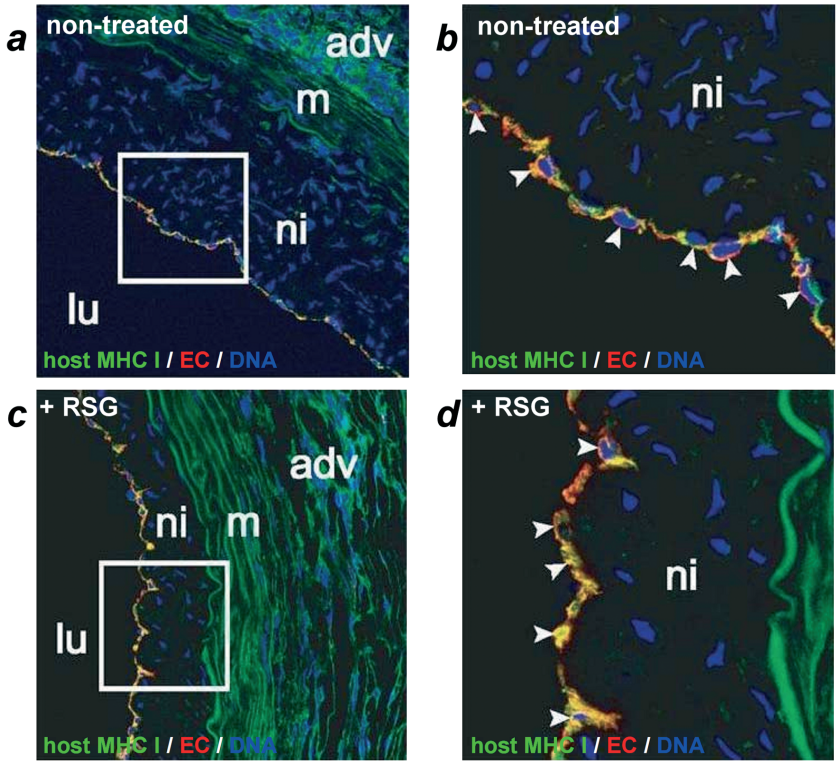

Figure 2. Neointimal EC`s are of host-origin in allografts from both RSG-treated and non-treated control rats. (**a**) Non-treated control allograft stained for host MHC-class I (mAb OX27, green), endothelium (mAb RECA-1, red) and DNA (DAPI, blue). The neointima is covered with a monolayer of host-derived endothelial cells (magnification x630). The adventitia is infiltrated with host-derived (green) inflammatory cells. (**b**) High-power magnification of the area within the white box shown in *a* (magnification x1890). (**c**) Also after RSG treatment, neointimal EC's are derived from the host as indicated by positive staining with OX27 and RECA-1. (**d**) High-power magnification of the area within the white box shown in *c* (magnification x1890). Arrowheads in *c* and *d* indicate host-derived EC's (positive with both OX27 and RECA-1, yellow). Note the autofluorescence (green) signal of the elastin fibers in the media in *a*, *c* and *d*. Abbreviations: adv: adventitia; lu: lumen; m: media; ni: neointima.

marker and recipient-MHC class I) in both the grafts from non-treated rats (Figure 2a,b) and RSG-treated rats (Figure 2c,d).

Rosiglitazone suppresses alloreactivity

PPARγ agonists are known to have anti-inflammatory properties.[5,15] RSG may attenuate TA by reducing alloreactive T cell responses resulting in reduced vascular wall damage. To analyze whether RSG indeed reduces alloreactivity mixed lymphocyte reactions (MLR's) were performed using BN-derived responder cells (*i.e.* splenocytes from RSG-treated and non-treated control allograft recipients) (MLR1). BN responder cells were stimulated with irradiated donor-type (Lew), third-party (WF) and syngeneic (BN) splenocytes or with ConA (5 μg/ml). As shown in Figure 3a, proliferative responses were significantly suppressed in cultures containing responder splenocytes derived from RSG-treated recipients ($p<0.05$ *vs.* non-treated controls). Proliferative responses were equally reduced in RSG-treated responder cells stimulated with donor-type (Lew) and third-party type (WF) allogeneic stimulator cells. No differences were observed between RSG-treated and non-treated control rats after polyclonal stimulation with ConA (Figure 3a).

In another set of experiments, MLR`s were performed using both Lew and BN responder cells obtained from naive (non-treated non-transplanted) rats that were stimulated with irradiated allogeneic stimulator cells (BN and Lew, respectively) in the presence or absence of 100 μM RSG. RSG markedly reduced the alloreactive proliferative response of both

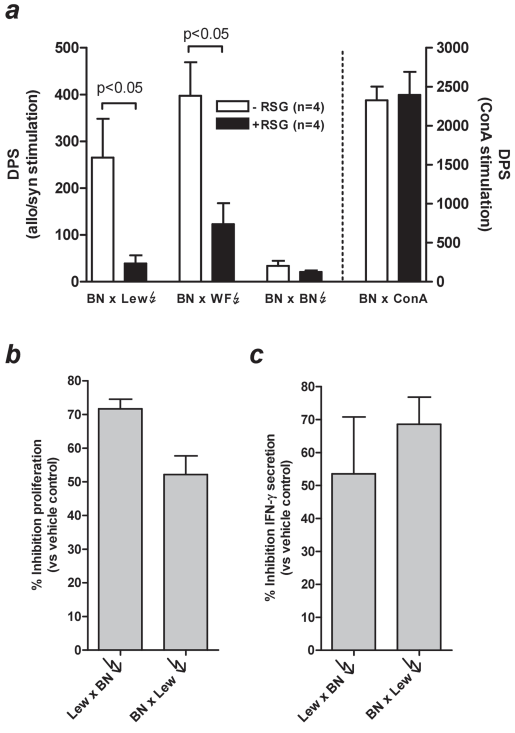

Figure 3. RSG inhibits *in vitro* **T cell alloreactivity.** (**a**) RSG treatment of graft recipients *in vivo* reduces the alloreactive response as measured in a one-way MLR *in vitro*. Splenocytes from non-treated control (white bars) and RSG-treated (black bars) allograft recipients were stimulated with 30 Gy irradiated (arrow) donor-type (Lew), third-party (WF) and syngeneic (BN) splenocytes or with ConA (5 μg/ml). Proliferative responses were significantly suppressed in cultures containing splenocytes derived from RSG-treated allograft recipients (black bars) ($p<0.05$, non-paired *t* test, *vs.* non-treated control allograft recipients, white bars). No differences were observed after ConA stimulation. (**b**) Adding RSG to the MLR *in vitro* suppresses proliferative alloreactive responses. Naive Lew and BN responder cells were stimulated with 30 Gy irradiated (arrow) allogeneic stimulator cells (BN and Lew splenocytes, respectively). Proliferative responses were markedly inhibited in the presence of 100 μM RSG. Data expressed as percentage inhibition relative to their vehicle controls. (**c**) INF-γ levels in culture supernatants were significantly reduced in the presence of 50 μM RSG ($p<0.05$, paired *t* test, *vs.* vehicle control). Data are presented as mean ± SEM.

Lew and BN responders (expressed as the percentage inhibition relative to their vehicle controls) (Figure 3b) accompanied by reduced levels of IFN-γ in the culture supernatants (Figure 3c, Lew x BN, $p<0.01$; BN x Lew, $p<0.05$, paired *t* test). In order to determine whether also ConA-induced responses can be suppressed after adding RSG (100 μM) to the cultures, BN responder cells were stimulated with ConA (10 or 2.5 μg/ml) in the presence of RSG or the vehicle. Proliferative responses after stimulation with 10 and 2.5 μg/ml ConA were significantly reduced by respectively 82% ($p=0.01$) and 77% ($p<0.0001$). These results indicate that RSG clearly suppresses ConA-induced proliferative responses but that the efficacy of suppression might depend on type (*in vivo vs. in vitro*) and concentration of exposure to RSG.

Rosiglitazone does not influence CD4+CD25+ FoxP3+ Treg cell frequency and function

To analyze whether reduced alloreactivity is associated with increased Treg frequency and function, we determined the frequency and absolute numbers of $CD4^+CD25^+FoxP3^+$ T

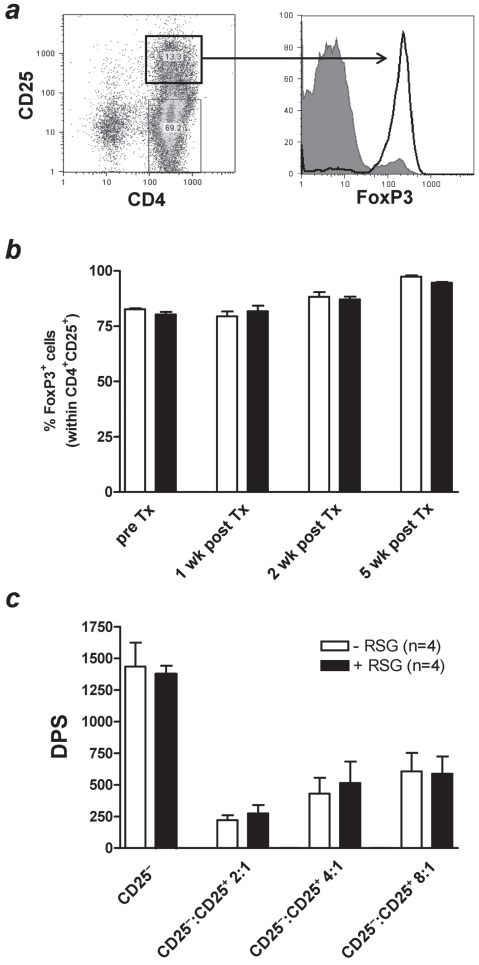

Figure 4. RSG treatment *in vivo* does not influence $CD4^+CD25^+FoxP3^+$ Treg frequency, absolute numbers and suppressor function. (**a**) FACS plots showing the gating profile used for generating the data as presented in Figure 4b and Table 1. Within the TcR^+ population (not shown) the $CD4^+CD25^+$ cells were gated (left panel). Within the $CD4^+CD25^+$ T cell population FoxP3 expression was analyzed (right panel). Filled histogram represents FoxP3 expression within the $CD4^+CD25^-$ T cell population. (**b**) The percentage FoxP3-expressing cells within the $CD4^+CD25^+$ T cell population increases in time (see also Table 1). However, no differences are present between the RSG-treated and non-treated control rats. (**c**) RSG treatment *in vivo* does not alter the suppressive characteristics of $CD4^+CD25^+$ T cells as measured in an *in vitro* co-culture suppression assay. In the co-cultures, fixed numbers of $1x10^5$ sorted $CD4^+CD25^-$ responder T cells (pooled cells isolated from 4 allografted, non-rosiglitazone treated BN rats) were cultured with $1x10^5$ irradiated APCs, ConcanavalinA (10 μg/ml), and different amounts of purified $CD4^+CD25^+$ Tregs. Tregs were isolated from allografted, non-treated control rats (white bars) or allografted, RSG-treated rats (black bars). The following $CD4^+CD25^-$: $CD4^+CD25^+$ ratios were tested: 2:1, 4:1 and 8:1. To test for direct effects of RSG on effector T cells, $CD4^+CD25^-$ T cells were isolated from both RSG-treated and non-treated BN rats and stimulated with ConcanavalinA in the presence of irradiated APC's but without adding $CD4^+CD25^+$ Tregs.

cells in the peripheral blood and the suppressor function of $CD4^+CD25^+$ T cells *in vitro*. Figure 4a shows the gating strategy used to determine the frequency of $CD4^+CD25^+FoxP3^+$ T cells in the peripheral blood. Aortic allografting resulted in a significant decrease in the percentage of $CD4^+CD25^+$ T cells 5 wks post-transplantation (Table 1, $p<0.05$, *vs.* pre-transplantation values). However, the absolute numbers of circulating $CD4^+CD25^+$ T cells significantly increased in time (Table 1, $p<0.05$, *vs.* pre-transplantation values). No differences in frequency and absolute numbers were observed between the non-treated controls and the RSG-treated rats. The increase in absolute numbers of $CD4^+CD25^+$ T cells is caused to great extent by increased numbers of $FoxP3^+$ cells within the $CD4^+CD25^+$ T cell subset (Table 1, $p<0.05$, 5 wks post-transplantation *vs.* pre-transplantation values). No differences in frequency and absolute numbers of $CD4^+CD25^+FoxP3^+$ T cell were observed between the non-treated controls and the RSG-treated rats (Table 1 and Figure 4b).

In addition to the frequency and absolute numbers of $CD4^+CD25^+FoxP3^+$ Tregs, also Treg function, as measured in an *in vitro* co-culture suppression assay, was not altered by *in vivo* exposure to RSG. Lymph node-derived $CD4^+CD25^-$ T cells obtained from both RSG-treated and non-treated control rats proliferated to the same extent in response to polyclonal stimulation with ConA indicating that the proliferative capacity of responder cells is not hampered after *in vivo* exposure to RSG. Lymph node-derived $CD4^+CD25^+$ T cells obtained from both RSG-treated and non-treated control rats were equally efficient in suppressing proliferation of ConA-stimulated $CD4^+CD25^-$ T cells in a cell dose-dependent manner (Figure 4c). Statistically significant suppression ($p<0.0001$, unpaired *t* test) was observed when $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were cocultured at ratios of 2:1, 4:1 and 8:1 (85%, 70% and 58% suppression respectively for non-treated control rat-derived $CD4^+CD25^+$ T cells, and 80%, 63% and 57% suppression respectively for RSG-treated rat-derived $CD4^+CD25^+$ T cells).

Rosiglitazone reduces intragraft SDF-1α and PDGFRβ gene expression

We previously demonstrated that neointimal VSMC's in aortic and cardiac allografts are recipient-derived and supposedly originate from a pool of circulating progenitor cells.[10,16-18]

Table 1. Frequency and absolute numbers of circulating $CD4^+CD25^+FoxP3^+$ Tregs

	- RSG				+ RSG			
Time after transplantation (wks)	**$CD25^+$**		**$FoxP3^+$**		**$CD25^+$**		**$FoxP3^+$**	
	% of $CD4^+$ T cells	**x10^5 (cells/ml)**	**% of $CD4^+$ $CD25^+$ T cells**	**x10^5 (cells/ml)**	**% of $CD4^+$ T cells**	**x10^5 (cells/ml)**	**% of $CD4^+$ $CD25^+$ T cells**	**x10^5 (cells/ml)**
pre Tx	16.4 ± 2.8	1.7 ± 0.8	82.6 ± 0.4	1.3 ± 0.7	20.1 ± 4.1	1.9 ± 0.9	80.2 ± 2.3	1.5 ± 0.7
1	13.9 ± 0.7	2.3 ± 0.5	79.4 ± 4.4	1.8 ± 0.4	14.0 ± 1.3	2.8 ± 0.4	81.7 ± 5.5	2.3 ± 0.4
2	10.8 ± 0.9	3.4 ± 0.7	88.2 ± 3.7	3.0 ± 0.7	11.0 ± 0.5 *	3.8 ± 0.8 *	87.0 ± 2.8 *	3.3 ± 0.7 *,**
5	12.1 ± 0.4 *	4.0 ± 0.3 *	97.3 ± 1.2 *,**	3.9 ± 0.3 *,**	11.7 ± 0.4 *,**	3.9 ± 0.3 *,**	94.6 ± 0.9 *,**,***	3.6 ± 0.3 *,**

($p<0.05$, * vs. pre Tx, ** vs. 1 wk, *** vs. 2 wk, Mann Whitney U test, n=3-4/group)

RSG may affect neointima development by reducing recruitment of VSMC progenitor cells to sites of vascular injury in the graft. To test this hypothesis we analyzed by real-time PCR the levels of intragraft expression of stromal-derived factor-1α (SDF-1α), a chemoattractant for $CXCR4^+$ stem cells known to be involved in TA development.[19] As shown in Figure 5a, RSG treatment significantly reduced expression levels of SDF-1α (>2.5-fold reduction *vs.* non-treated controls, $p<0.05$, unpaired *t* test). Growth factors, in particular platelet-derived growth factor (PDGF) and their receptors (PDGFR`s) are major players implicated in VSMC migration and proliferation and subsequent neointima formation.[20] We therefore analyzed intragraft PDGFRβ expression levels after RSG-treatment. As shown in Figure 5b, treatment of allograft recipients with RSG resulted in significantly reduced intragraft PDGFRβ expression levels (>8-fold reduction, *vs.* non-treated controls, $p<0.05$, unpaired *t* test). Similar results were obtained when analyzing PDGFRβ expression levels in isolated neointimal tissue (>14-fold reduction, *vs.* non-treated controls, $p<0.05$, Mann Whitney *U* test, data not shown).

4

Rosiglitazone inhibits PDGF-BB induced VSMC proliferation

As described above, RSG-treatment *in vivo* reduced intragraft expression levels of (neointimal) PDGFRβ. To analyze whether RSG has a direct effect on VSMC proliferation both medial and neointimal VSMC`s were isolated and *in vitro* stimulated in the presence of PDGF-BB, a ligand for PDGFRβ and a strong inducer of VSMC proliferation. As shown in Figure 6a, 100 μM RSG significantly inhibits PDGF-BB–induced medial (Lew: 78±22% *vs.* vehicle control; BN: 65±12% *vs.* vehicle control) and neointimal (Lew to BN: 85±5% *vs.* vehicle control) VSMC proliferation (black bars, $p<0.05$, *vs.* vehicle controls, paired *t* test). To test whether this inhibitory effect of RSG on medial and neointimal VSMC proliferation is mediated through PPARγ, VSMC's were stimulted with PDGF-BB in the presence of RSG and a specific irreversible PPARγ antagonist (GW9662). Pretreatment with GW9662 (5 μM) did not abrogate the inhibitory effect of RSG (Figure 6a, grey bars). To demonstrate that the PPARγ-antagonist GW9662 was effective in the assay system used, VSMC (both medial and neointimal) proliferation was determined after PDGF-BB stimulation in the presence of GW9662 (5 μM). Blocking PPARγ resulted in increased proliferative responses in the different VSMC's tested (data not shown). These data indicate that the PPARγ antagonist

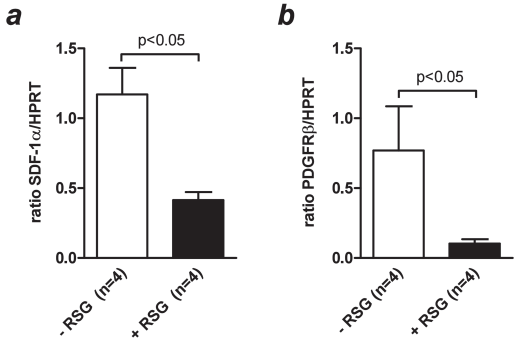

Figure 5. RSG treatment reduces intragraft SDF-1α (a) and PDGFRß (b) mRNA expression levels 6 wks after transplantation. mRNA expression levels are expressed relative to the expression of the house-keeping gene HPRT. Data are expressed as mean ± SEM, $p<0.05$, unpaired *t* test.

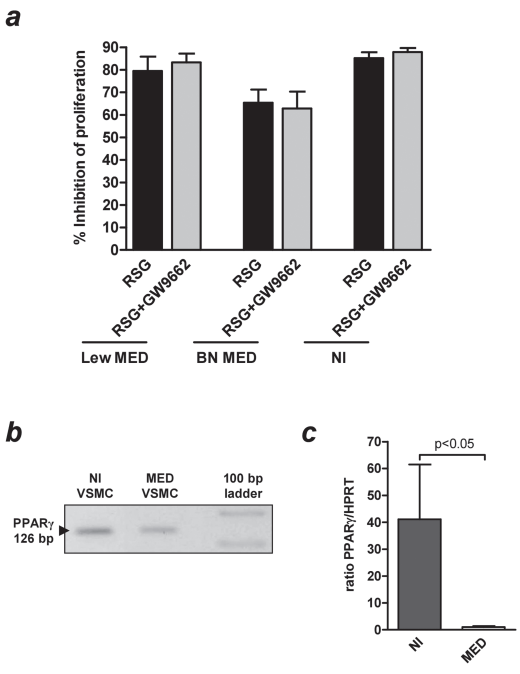

Figure 6. RSG inhibits PDGF-BB-induced medial and neointimal VSMC proliferation in a PPARγ-independent manner. (**a**) Medial and neointimal VSMC`s ($1.5x10^4$ cells/well) were seeded, allowed to attach and serum-starved. Next, cells were stimulated for 48 hrs with PDGF-BB (25 ng/ml). Stimulation was performed in the presence or absence of 100 μM RSG (or 1% EtOH) and in the presence or absence of the PPARγ antagonist GW9662 (5 μM) (or 0.05% DMSO). PPARγ antagonist GW9662 and RSG were added to the cells respectively 60 and 30 min prior to adding the VSMC mitogen PDGF-BB. Cells were stimulated for 48 hrs and 0.5 μCi [^{3}H]-thymidine/well was present during the last 24 hrs of culture. Proliferation was measured by liquid scintillation and results are expressed as the percentage inhibition compared with the appropriate vehicle controls. 100 μM RSG significantly inhibits PDGF-BB–induced medial and neointimal VSMC proliferation (black bars, $p<0.05$, *vs.* vehicle controls, paired *t* test). Similar percentage inhibition was observed in the presence of the PPARγ antagonist GW9662 (grey bars). Data from four independent experiments are shown and presented as mean ± SEM. (**b**) PPARγ mRNA expression was detected in medial and neointimal VSMC's by RT-PCR. **(c)** Real-time RT-PCR performed on medial (BN and Lew, n=3) and neointimal (Lew to BN allografts, n=5) VSMC's. mRNA expression levels are expressed relative to the expression of the house-keeping gene HPRT. Data are expressed as mean ± SEM, $p<0.05$, Mann Whitney *U* test. Abbreviations: MED: medial; NI: neointimal.

GW9662 is effective in our assay system and abbrogates the inhibitory effect of PPARγ ligation by endogenous molecules. Both medial and neointimal VSMC's express PPARγ (Figure 6b) with increased expression levels being observed in neointimal VSMC's (Figure 6c). Despite the presence of the ligand for RSG on VSMC's, our proliferation data indicate that the inhibitory effect of RSG on VSMC proliferation is not mediated through PPARγ.

Discussion

Although currently used immunosuppressive regimens are highly effective in preventing acute rejection, there is no adequate therapy available to treat or prevent chronic transplant dysfunction (CTD). The common histopathological hallmark of CTD is transplant arteriosclerosis (TA), *i.e.* the formation of an occlusive neointima in intragraft arteries resulting from uncontrolled proliferation of VSMC's .[2,3]

Thiazolidinediones are PPARγ agonists and represent a relatively new class of oral agents that are clinically used to improve insulin resistance in Type 2 diabetics.[4,5] However, increasing amounts of experimental and clinical data point towards a beneficial role of PPARγ agonists in attenuating vascular remodeling and acting as an anti-inflammatory agent.[9,21-26] PPARγ is a ligand-activated transcription factor belonging to the nuclear

receptor family and is expressed by all cells involved in vascular remodeling *i.e.* VSMC's, EC's, macrophages and T cells.[26-28] The paramount role of VSMC's in the development and progression of neointima formation was our rational for considering this cell population as a key target of PPARγ agonists to attenuate TA. We confirmed previous data showing that both medial and neointimal VSMC`s express PPARγ with increased expression levels in neointimal VSMC's[29] which may represent a defense mechanism of neointimal VSMC's to self-limit growth.[30] Despite this over-expression of PPARγ, neointima formation is a remodeling process that goes beyond the needs of functional repair and therefore requires therapeutical intervention. In this study we tested the hypothesis that the PPARγ agonist rosiglitazone (RSG) is efficacious in attenuating TA development after allogeneic aorta transplantation in rats.

Our study demonstrates that RSG treatment in transplanted rats presented with significantly reduced neointima formation. The advantage of using the aorta transplant model rather than a solid organ transplantation model is the fact that after allogeneic aorta transplantation no anti-rejection therapy is required to prevent a full-blown acute rejection response.[10] Consequently, by using aorta transplantation the observed effects of RSG on neointima formation can be solely attributed to direct effects of RSG. Analysis of RSG plasma levels showed presence of RSG in the circulation which was biologically active as demonstrated by increased intragraft CD36 mRNA expression in RSG treated rats, a well-characterized downstream target in the PPARγ signalling cascade[31] (see *Supplementary Data*).

To understand the underlying mechanism of RSG-induced reduction of neointima formation, we first determined the origin (graft *vs.* recipient) of EC's that covered the luminal surface of the neointima in the aortic allografts. We previously showed endothelial cell replacement with host-derived endothelium (*i.e.* EC chimerism) after aortic allografting[10,16] reflecting severe vascular injury rather than graft adaptation.[17,32] Although RSG does clearly not preserve graft endothelium, it may still enhance vascular repair by increasing reendothelialization of the denuded graft vascular wall which is currently under investigation. In line with this, the PPARγ agonist troglitazone is reported to stimulate endothelial repair after balloon catheter injury.[33] Enhanced endothelial repair is furthermore supported by our observation that RSG treatment restored severe systemic endothelial dysfunction that was induced after allogeneic aorta transplantation (data not shown).

As thiazolidinediones are anti-inflammatory[5,7,8,15,34], we analyzed the allogeneic T cell response of RSG-treated allografted rats *in vitro*. Splenocytes from RSG-treated rats showed significantly reduced proliferative responses against both donor-type and third-party allogeneic irradiated stimulator cells. Moreover, RSG added *in vitro* to MLR's with naive responder splenocytes significantly suppressed their proliferative response against allogeneic cells. The mechanism of RSG-induced attenuated alloreactivity is not fully understood but may include transrepression of proinflammatory genes involved in T cell expansion (*e.g.* IL-2)[5,7] and skewing the immune response towards a Th2-type response.[35] In line with the latter mechanism, we indeed showed reduced secretion of the Th1-type cytokine INF-γ after adding RSG to the MLR cultures. Furthermore, RSG treatment *in vivo* might reduce the alloreactive response through the induction of $CD4^+CD25^+FoxP3^+$

regulatory T cells (Tregs).[36] PPARγ has recently been shown to be involved in both downregulation of $CD4^+$ T effector cell function as well as increasing Treg suppressor function in a colitis mouse model.[37] Furthermore, treatment of murine $CD4^+CD25^-FoxP3^-$ effector T cells with the PPARγ-agonist ciglitazone *in vitro* enhanced the conversion towards induced $CD4^+CD25^+FoxP3^+$ Tregs. Moreover, treatment with ciglitazone *in vivo* reduced severity of disease in a model of murine graft-vs-host disease which was mediated by naturally occurring $CD4^+CD25^+FoxP3^+$ Tregs in a PPARγ-dependent manner.[38] These recent studies indicate that triggering of PPARγ expressed on Tregs either by endogenous ligands or by a synthetic PPARγ ligand results in enhanced Treg function. In our study aorta transplantation resulted in significantly increased numbers of $CD4^+CD25^+FoxP3^+$ Tregs several weeks after transplantation. However, no differences in numbers and function of $CD4^+CD25^+FoxP3^+$ Tregs were observed after *in vivo* treatment with RSG. Although our *in vitro* results suggest that Tregs are not key players in reducing the alloreactive response after exposure to RSG we can not exclude the possibility that naturally occurring $CD4^+CD25^+FoxP3^+$ Tregs are involved *in vivo* but without modulation of Treg numbers as recently reported.[38]

Besides its suppressive effect on alloreactivity RSG might also act directly on the major cell type involved in neointima formation *i.e.* VSMC's. It is now well established that the majority of VSMC`s forming neointima in aortic allografts are recipient–derived.[10] Therapeutic targeting of recruitment, homing, differentiation and proliferation of VSMC progenitor cells into the injured allograft vascular wall is therefore a feasible approach to limit TA. Stromal-derived factor-1α (SDF-1α) is a chemoattractant for stem cells and its neutralization in a murine aortic transplant model was shown to inhibit mobilization of hematopoietic stem cells and reduced neointima formation.[19] In line with this, we observed reduced intragraft expression of SDF-1α after RSG-treatment that may have caused reduced recruitment of VSMC progenitor cells towards the injured vascular wall and consequently attenuated TA.

Also direct anti-proliferative effects of RSG on neointimal VSMC's may have contributed to reduced neointima formation.[39] Platelet-derived growth factors and their receptors play a central role in driving VSMC proliferation in the expanding neointima.[20] In our experiments RSG significantly reduced neointimal expression levels of PDGFRβ. Also *in vitro* RSG suppressed PDGF-induced proliferation of both rat medial and neointimal VSMC`s. Antiproliferative effects of thiazolidinediones are reportedly mediated via both PPARγ-dependent[29,40] and independent mechanisms.[41-43] Using a specific PPARγ antagonist we showed that RSG does not suppress medial and neointimal VSMC proliferation through PPARγ.

In this study we thus show that RSG-treatment attenuates neointima formation by affecting various targets known to be involved in the pathophysiology of TA *i.e.* inhibition of the alloreactive response and inhibition of neointimal VSMC proliferation and reduced expression of SDF-1α which is known to be involved in recruitment and homing of progenitor cells. Since development of TA, together with endothelial dysfunction, are major players in the increased morbidity and mortality among transplant recipients, treatment

with PPARγ agonists, in addition to the conventional immunosuppressive regimen, may offer beneficial effects on TA development and associated complications. This is supported by the observation that RSG can be given for a relatively short period (1-10 months) to renal transplant recipients to treat insulin resistance and endothelial dysfunction without serious side effects.[44,45] We therefore propose that RSG offers new treatment opportunities to tackle today's major problem in transplant biology for which no adequate treatment options are available yet.

Acknowledgments

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4

Supplementary data

Methods

Mixed lymphocyte reaction

To analyze the effect of rosiglitazone on T cell alloreactivity two different experimental setups were used. In the first setup (MLR 1), alloreactive T cell responses of rats that were RSG-treated *in vivo* (or left untreated) were measured in an *in vitro* MLR. In the second setup, MLR's were performed with splenocytes obtained from naive rats (no RSG treatment, no transplantation) and RSG was added to the cultures (MLR 2). **MLR 1:** Splenocytes were isolated from RSG-treated allograft-recipients 8 wks after transplantation. Spleens were removed and single cell splenocyte suspensions were obtained by gentle extrusion through stainless steel sieves. Suspensions were adjusted to a concentration of $2x10^6$ cells/ml in culture medium (RPMI1640 supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM L-glutamin, 60 µg/ml gentamycin, 1 mM sodium pyruvate and 4 µM β-mercaptoethanol) after lysis of erythrocytes in NH_4Cl buffer. As stimulator cells splenocytes from syngeneic (BN), allograft donor (Lew) and third-party allogeneic WF rats were used. Stimulator cells were treated with NH_4Cl buffer and subsequently exposed to 30 Gy γ-irradiation (^{137}Cs, IBL673, CIS Bio International, France). Irradiated cells were resuspended in culture medium at $4x10^6$ cells/ml. For stimulation, $4x10^5$ responder cells and $4x10^5$ irradiated stimulator cells were delivered in quadruplicate in 96-well round-bottom tissue culture plates. Cells were cocultured for 80 hrs at 37°C under 5% CO_2. As a positive control for proliferation, responder cells were stimulated with ConcanavalinA (5 µg/ml). **MLR 2:** Splenocyte suspensions were obtained from naive BN and Lew rats as described above. A total of $2x10^5$ responder cells (Lew or BN) and $4x10^5$ irradiated stimulator cells (BN or Lew, respectively) were cocultured in 96-well plates at 37°C under 5% CO_2 in the presence or absence of rosiglitazone (kindly provided by GlaxoSmithKline, Zeist, The Netherlands). Lew and BN responder cells were cultured for 4 and 5 days, respectively. These

were the optimal conditions to analyze alloreactive T cell responses in the Lew and BN rat strains.) Rosiglitazone (final concentration 100 μM) or vehicle (1% EtOH) were added to the responder cells, 30 minutes prior to adding the stimulator cells. Proliferation was assessed by liquid scintillation and results were expressed as disintegrations per second (dps) (MLR1) or as percentage inhibition of proliferation relative to the vehicle control (MLR2).

Flowcytometric analysis

To determine the frequency and absolute numbers of $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood of transplanted rosiglitazone-treated and non-treated rats, flowcytometric analyses were performed on peripheral blood mononuclear cells (PBMNC`s) at the following time-points: pre-transplantation and 1 wk, 2 wks and 5 wks post-transplantation. PBMNC`s were isolated from buffy coats obtained from heparinized peripheral blood collected by cardiac puncture. Erythrocytes in the PBMNC's were lysed with hypotonic NH_4Cl buffer. PBMNC's ($2x10^6$ cells) were reacted with a mixture of anti-αßTcR biotin (clone R73), anti-CD4 FITC (clone OX-35) and anti-IL-2 receptor α chain PE (CD25, clone OX-39) monoclonal antibodies (BD Pharmingen, San Diego, CA, USA) for 20 min. at 4°C. Biotin was detected with Streptavidin PE-Cy5 (eBioscience, San Diego, CA, USA). Cell surface staining was followed by intracellular FoxP3 staining using anti-FoxP3 allophycocyanin (clone FJK-16s) and the eBioscience Foxp3 Staining Buffer Set (both from eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Cells were analyzed using a BD FACSCalibur (Becton Dickinson, Alphen aan den Rijn, The Netherlands). A minimum of 200,000 events was acquired for each analysis. The lymphocyte fraction was gated on the basis of forward and side scatter. The FlowJo software package (Tree Star Inc., Ashland, OR, USA) was used for analysis.

Isolation of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cells for *in vitro* proliferation assays were obtained from pooled mesenteric lymph node (MLN) and cervical lymph node (CLN) single cell suspensions from transplanted rosiglitazone-treated or non-treated BN rats (6 wks after transplantation). Lymph node-derived $CD4^+$ T cells from individual rats were negatively selected using rat CD4 Cell Recovery Columns (Cedarlane, Ontario, Canada) according to the manufacturer's instructions. The purity of the $CD4^+$ T cells obtained by this method was measured by flow cytometry and was consistently >97%. Purified $CD4^+$ T cells were incubated with PE-conjugated anti-CD25 (clone OX39) mAb for 30 min. Incubations were performed in 4 ml tubes on ice ($2x10^8$ cells/ml). $CD4^+CD25^-$ T cells (from transplanted, non-treated BN rats) and $CD4^+CD25^+$ T cells (from transplanted, treated or non-treated BN rats) were sorted using a MoFlo® high-speed cell sorter (DakoCytomation B.V., Heverlee, The Netherlands).

APC preparation

As antigen presenting cells (APC's) in *in vitro* proliferation assays BN splenocytes were used. Single cell suspensions were prepared as described above and adjusted to a concentration

of 2x10^6/ml after lysis of erythrocytes in hypotonic NH_4Cl buffer and 30 Gy γ-irradiation (^{137}Cs, IBL673, CIS Bio International, France).

Regulatory T cell suppression assays

Cultures were performed in RPMI1640 (supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM L-glutamin, 60 μg/ml gentamycin, 1 mM sodium pyruvate and 4 μM β-mercaptoethanol). Fixed numbers of 1x10^5 sorted CD4$^+$CD25$^-$ responder T cells (pooled cells isolated from four allografted, non-rosiglitazone treated BN rats) were cocultured in triplicate in 96-well plates with 1x10^5 autologous irradiated APC's, ConcanavalinA (10 μg/ml), and different amounts of purified CD4$^+$CD25$^+$ Tregs (isolated from either allografted, non-rosiglitazone treated or allografted, rosiglitazone-treated BN rats). The following CD4$^+$CD25$^-$: CD4$^+$CD25$^+$ ratios were tested: 2:1, 4:1, 8:1, and CD4$^+$CD25$^-$ T cells alone. Cells were cultured for 114 hrs at 37°C in an atmosphere of 5% CO2 and 95% air. Cultures were pulsed with 1 μCi [3H]thymidine/well for the last 24 hrs of culture. Proliferation in each independent experiment was assessed by liquid scintillation and the disintegrations per second (dps) of each triplicate assay were averaged.

Rat medial and neointimal VSMC proliferation

Medial VSMC`s from thoracic aorta's of Lew and BN rats were isolated using enzymatic digestion as described.[1] Peri-aortic tissue was removed from freshly collected thoracic aorta's which were then cut longitudinally. Endothelium was mechanically removed using a cotton swab. Under a dissecting microscope, tunica media was separated from adjacent adventitia and cut into small pieces of ~1 mm^2. For cell dispersion, medial muscle strips were treated with collagenase (3.5 mg/ml), elastase (1 mg/ml), soybean trypsin inhibitor (0.1 mg/ml) and DNA-ase (0.05 mg/ml) (all from Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands) in successive incubation steps at 37°C in a rocking water bath.

For neointimal VSMC isolation, abdominal aorta transplantation was performed in the Lew-to-BN strain combination. Grafts were explanted 10 wks post-transplantation, cut longitudinally after which the endothelium was gently removed using a cotton swab. Using forceps, the neointima was carefully dissected from the adjacent media and minced in small pieces followed by enzymatic dispersion as described above. Both medial and neointimal VSMC`s were cultured in EMEM supplemented with 4 mM glutamin, 100 mM non-essential aminoacids (all from Invitrogen, Breda, The Netherlands), 10% FBS (Perbio Science Nederland B.V., Etten-Leur, The Netherlands) and 50 μg/ml gentamycin. Cells were identified as VSMC`s based on their morphology (*i.e.* typical "hill and valley" morphology and positive staining for α-smooth muscle actin) and growth characteristics as reported previously.[1] Both early and late passages were used (between passage 2 and 61) with similar results. To determine cell proliferation, medial and neointimal VSMC`s were plated onto 96-well flat bottom culture plates at 1.5x10^4 cells/well and were allowed to attach for 24 hrs. Cells were then serum-starved for 24 hrs in medium containing 0.4% FBS. Subsequently, cells were stimulated for 48 hrs with 25 ng/ml PDGF-BB (R&D Systems, Europe Ltd., Abingdon, UK) in the presence of 100 μM rosiglitazone or 1% EtOH (vehicle control).

Rosiglitazone was added 30 minutes before adding PDGF-BB. To analyze whether VSMC proliferation is modulated by rosiglitazone through PPARγ, cells were pretreated with the PPAR-γ antagonist GW9662 (Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands). The antagonist (final concentration 5 μM) or vehicle (0.05% DMSO) were added 30 min before adding rosiglitazone. After culturing the cells for the last 24 hrs in the presence of 0.5 μCi/well [^{3}H]-thymidine, cells were trypsinized and harvested on glass filters. Proliferation was assessed by liquid scintillation and results were expressed as the percentage inhibition of proliferation compared with the appropriate vehicle control.

Results

Rosiglitazone plasma levels

As rosiglitazone (RSG) was admixed in the diet, drug plasma levels were determined. Eight weeks after transplantation RSG plasma levels were 2.2±0.3 μg/ml in treated animals (n=8) and below detection levels in non-treated rats (n=6) (Figure 1a). These results indicate that RSG was metabolized and entered the circulation. To analyze whether RSG was also biologically active and triggered PPARγ, intragraft CD36 (fatty acid translocase) mRNA expression was determined in aortic allografts. CD36 is among other cell types expressed by vascular cells[2] and is one of the target genes located downstream of PPARγ.[3] At 8 weeks post-transplantation CD36 mRNA expression was significantly increased in allografts from RSG-treated rats (>2.4-fold increase, p<0.05, *vs.* non-treated controls) (Figure 1b).

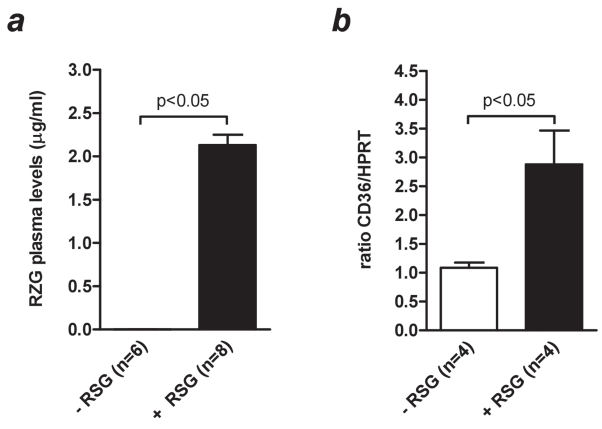

Figure 1. Rosiglitazone (RSG) is present in the circulation and activates PPARγ. (**a**) After oral administration (admixed in the chow) RSG is detected in the plasma of treated rats (black bar) whereas RSG levels were below detection level in the non-treated control group. Plasma was collected at the time of sacrifice (8 wk post-transplantation) and RSG levels measured by high-pressure liquid chromatography with tandem mass spectrometry. (**b**) RSG treatment (black bar) results in increased CD36 mRNA expression indicative of PPARγ activation. Data are expressed as mean ± SEM, p<0.05 *vs.* non-treated controls, Mann Whitney *U* test.

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Table 1. Primers used for PCR analysis

Primer	Sequence (5`- 3`)	$T_{Annealing}$ (°C)	Amplicon size (bp)	Genbank Accession nr
HPRT	Fw: GCG AAA GTG GAA AAG CCA AGT	60	76	NM012583
	Rev: GCC ACA TCA ACA GGA CTC TTG TAG			
CD36	Fw: CAT GCA AGT CCT GAT GTC TC	60	150	NM031561
	Rev: TCT AGC TGG CTT GAC CAG TA			
SDF-1α	Fw: TTG CCA GCA CAA AGA CAC TCC	60	226	AF209976
	Rev: CTC CAA AGC AAA CCG AAT ACA G			
PDGFRβ	Fw: TGC TCA CCA CCT CAT ATT CC	60	158	NM031525
	Rev: TGC CTC AGC CAA ATG TCA CC			
PPARγ	Fw: CAT TGG GTC AGC TCT TGT GA	60	126	NM013124
	Rev: TGT GGA CCT CTC TGT GAT GG			

5

Dichotomous effects of rosiglitazone in transplantation-induced systemic vasodilator dysfunction in rats

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Abstract

Background. Transplantation-induced systemic endothelial dysfunction causes severe cardio-vascular morbidity and mortality after transplantation. Interventions that improve systemic endothelial function after transplantation and furthermore reduce intragraft vascular dysfunction might improve graft and patient survival. Treatment with the PPARγ agonist rosiglitazone is an intervention that potentially fulfills these criteria. In this study we determined the effect of rosiglitazone treatment on transplantation-induced endothelial dysfunction and vasomotor activity in an experimental model for chronic transplant dysfunction in rats.

Methods. Lewis abdominal aortic allografts were orthotopically transplanted into Brown Norway recipients that received either regular chow or chow containing rosiglitazone (~4.2 mg/day). Endothelium-dependent (response to metacholine) and total (response to sodium nitrite) vasodilatory responses were determined in autologous thoracic aortic rings using an *ex vivo* organ bath setup. Measurements were performed 8 weeks after transplantation.

Results. Aortic allografting induced systemic endothelial dysfunction as measured by reduced endothelium-dependent vasodilation in the recipient's vascular system. Rosiglitazone treatment restored endothelium-dependent vasodilatory responses to pre-transplantation levels. However, rosiglitazone treatment reduced the total dilatory response despite normalized endothelial function, indicating impairment of vascular smooth muscle cell vasomotor activity.

Conclusions. Rosiglitazone treatment after allogeneic transplantation restores endothelial function but impairs vascular smooth muscle cell vasomotor activity. This dichotomous effect of rosiglitazone might impede use of rosiglitazone after organ transplantation since this potentially increases cardiovascular risk despite improved endothelial cell function.

Abbreviations

CVD	cardiovascular disease	**RSG**	rosiglitazone
EC	endothelial cell	**SED**	systemic endothelial dysfunction
ME	metacholine		
PE	phenylephrine	**SN**	sodium nitrite
PPAR-γ	peroxisome proliferator-activated receptor-γ	**VSMC**	vascular smooth muscle cell

Introduction

After solid organ transplantation vascular dysfunction is an important problem, both within and outside the transplanted organ. Within the transplanted organ, transplant arteriosclerosis is a main contributor to transplant dysfunction, as has been shown for the heart[1] and the kidney.[2] The development of transplant arteriosclerosis is supposed to cause downstream ischemic tissue damage resulting in deterioration of graft function and graft loss eventually. Outside the transplanted organ, transplant-associated systemic endothelial dysfunction (SED) occurs, which is a main contributor to the elevated cardiovascular risk in transplant recipients. In renal transplantation, recipients are at increased risk to develop cardiovascular disease (CVD) which is associated with reduced patient survival independent of graft dysfunction.[3] CVD in this population is multifactorial. By the time of transplantation most patients have established cardiovascular damage, related to pre-existent CVD as well as to the complex of factors associated with uremia.[4] After transplantation uremia-related cardiovascular risk factors improve, but factors related to the transplanted state emerge. Among the latter, an important factor is new-onset diabetes mellitus, which is characterized by insulin-resistance and SED.[3,5] SED in transplant recipients is associated with significant morbidity and mortality.[6-8] SED after transplantation develops not only as a result of the pre-existent clinical condition, but also as a result of the systemic inflammatory burden caused by the ongoing, subclinical, rejection response.[9]

Peroxisome proliferator-activated receptors (PPAR's) are ligand-activated transcription factors that belong to the nuclear receptor family that modulate expression of genes involved in lipid and glucose metabolism.[10] Thiazolidinediones, such as rosiglitazone (RSG), are synthetic agonists of PPARγ, a specific subfamily of PPAR's. Thiazolidinediones are prescribed to Type 2 diabetics as anti-hyperglycaemic drugs by virtue of their insulin-sensitizing effects.[11] Since PPARγ is also expressed on vascular smooth muscle cells (VSMC's) and endothelial cells (EC's)[11,12], treatment with PPARγ agonists modulate vascular biological processes.[13] PPARγ agonists have been shown to improve vasodilator function under conditions associated with SED such as hypertension[14,15], diabetes[16], and metabolic syndrome.[17,18] Furthermore, PPARγ agonists have been shown to attenuate intima hyperplasia (restenosis) after coronary stenting in both diabetic and non-diabetic patients.[19-22] Reduced VSMC migration and proliferation might be the underlying mechanism of reduced neointima formation following treatment with PPARγ agonists.[23] These clinical data have been confirmed in animal experimental models of restenosis in (non) diabetic rodents.[24-26] Since VSMC's are the main constituents of neointimal lesions in both restenosis and transplant arteriosclerosis, we recently tested the efficacy of RSG to attenuate the development of transplant arteriosclerosis after experimental aorta transplantation in rats.[27] Our data demonstrate that RSG is indeed a very effective drug to reduce the development of transplant arteriosclerosis after allogeneic transplantation. Mechanisms involved include suppression of the alloreactive immune response as well as direct antiproliferative effects on VSMC's.[27] Similar results were reported after treatment

with RSG and pioglitazone after experimental heart transplantation in rats and mice, respectively.[28,29]

Treatment with RSG thus attenuates development of transplant arteriosclerosis and furthermore has been shown to improve insulin resistance after renal transplantation.[30,31] We now propose that RSG might have potential to additionally improve SED after transplantation independent of improving insulin resistance. Together these effects of RSG can be anticipated to reduce cardiovascular risk and thereby patient morbidity and mortality. However, a recent widely publicized meta-analysis published by Nissen *et al.* concluded that treatment with RSG significantly increased risk for myocardial infarction and cardiovascular death.[32] Although this study has some limitations[33], increased risk of myocardial infarction and heart failure in Type 2 diabetics treated with rosiglitazone was confirmed by a meta-analysis performed by Singh *et al.*[34] However, others could not confirm these data[35,36] and therefore the precise effect of RSG on cardiovascular risk needs as yet to be established.

The potential hazard of RSG on increasing cardiovascular risk led us to test the effect of RSG on transplantation-induced SED and vasomotor activity. To this end we determined the endothelial (EC)-dependent and total vasomotor activity (that comprises an endothelium-dependent and an endothelium-independent VSMC response) in an experimental model of chronic transplant dysfunction and transplant arteriosclerosis *i.e.* aorta allografting in rats. We show that long-term treatment with RSG in transplanted rats causes an overall net improvement of vasodilator function outside the transplant. Although RSG treatment restored EC function to pre-transplantation levels, RSG also has direct deleterious effects on VSMC contractility and dilatory capacity. These dichotomous effects of RSG on EC function and VSMC vasomotor activity might impede use of RSG in transplantation as a treatment for SED, CVD and long-term allograft loss.

Methods

Rats

Specified pathogen free male Lewis (Lew, RT-1^{l}) and Brown Norway (BN, RT-1^{n}) rats were obtained from Harlan (Horst, The Netherlands). Rats were 13 weeks of age and were maintained under clean conventional conditions. Animals were housed and treated in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and institutional guidelines.

Surgical procedures

Under anesthesia (2% halothane, 0.4 L/min O_2 and 0.4 L/min N_2O) Lew aortic allografts were transplanted to BN recipients as described previously.[37] Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor, perfused with saline and subsequently orthotopically transplanted into the recipient via end-to-end anastomosis. Total cold and warm ischemic time was consistently less than 25 minutes. Recipient rats did not receive anti-rejection therapy after transplantation.

Rosiglitazone (RSG) treatment

Recipient rats were maintained on standard rat chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) formulated with or without RSG (Avandia, GlaxoSmithKline, Zeist, The Netherlands). RSG was admixed in the chow to a final concentration of 0.026% wt/wt. In this study we aimed at treating the rats (having a mean BW of ~250 gr during the treatment period) with a concentration of ~15 mg RSG/day/kg BW *i.e.* ~3.75 mg RSG/rat/day. This concentration was chosen since it was shown previously that treatment with 10-30 mg/RSG/day/kg BW is biologically active and results in normalization of HbA1c levels and significant lowering of plasma glucose and trigliceride levels in Zucker diabetic fatty (ZDF) rats treated with RSG for 8 weeks.[38] Based on a measured average food intake of ~16 g/day/rat, the average RSG intake in our experiments was ~4.2 mg/day/rat which was close to our target concentration. Food intake between RSG-treated and non-treated rats did not differ throughout the duration of the experiment. Treatment with RSG started 1 wk before transplantation and was continued throughout the duration of the experiment (8 weeks). Using this protocol RSG plasma levels obtained were 2.2±0.3 µg/ml in treated animals and below detection levels in non-treated rats.[27] Although the dose RSG per kg BW given to the rats was ~200x higher than the dose given to Type 2 diabetic patients (generally 4-8 mg/day), the peak plasma levels of RSG obtained in our experiments were 'only' 19x higher than the peak levels observed in healthy control subjects given a single oral dose of 8 mg RSG (peak levels: 117 ng/mL; http://www.gsk.com/products/prescription_medicines/ us/medicines-ae.htm; accessed November 6, 2007) indicating a difference in pharmacokinetics of RSG between rats and humans.

Measurement of vascular function

During sacrifice, the thoracic aorta was carefully removed for further preparation *ex vivo*. Peri-aortic tissue was removed from the thoracic aorta and rings of approximately 2 mm length were cut. The rings were connected to an isotonic displacement transducer at a preload of 14 mN in an organ bath with Krebs solution (pH 7.5) containing (mM): NaCl (120.4), KCl (5.9), $CaCl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), glucose (11.5), $NaHCO_3$ (25.0), at 37˚C and continuously gassed with 95% O_2 and 5% CO_2. After stabilization, during which regular washing was performed, rings were checked for viability by stimulation with phenylephrine (PE: 10^{-6} M). After viability check, the rings were washed and restabilized. Sets of rings were then contracted with cumulative doses of PE (10^{-9} to 10^{-6} M). After reaching the maximal contraction on PE, the EC-dependent vasodilation was assessed by cumulative doses of metacholine (ME: 10^{-8} to 10^{-4} M). Subsequently, the total dilatory response (that comprises an endothelium-dependent and an endothelium-independent VSMC response) was assessed by measuring the response to sodium nitrite (SN: 10 mM). PE and ME were purchased from Sigma-Aldrich (Steinheim, Germany). Salts and carbohydrates were purchased from Merck (Darmstadt, Germany). From each rat the responses of two aorta rings were measured and results obtained were averaged. No major differences were observed between the responses of the two rings.

Statistics

Vascular responses were expressed either in μm or in percentage from maximal contraction to PE or SN. The SN response in μm was calculated as maximal contraction to PE minus dilation to 10 mM SN. SN responses were also expressed as percentage of maximal PE response. E_{max} is the maximal response to PE or SN. Data are expressed as mean value ± standard error of the mean (SEM). Statistical analysis between dose-response curves were tested by General Linear Model (GLM) for Repeated Measures (and, where appropriate, corrected for sphericity: Greenhouse-Geisser correction). Single factor data were compared by Student's *t*-test. Values of $p<0.05$ were considered statistically significant. For statistical analysis SPSS 12.0.2 for Windows (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 4 for Windows (GraphPad Software, Inc., San Diego, CA, USA) was used.

Results

Rosiglitazone attenuates neointima formation after aortic allografting

Aortic allografts transplanted to BN recipients were characterized by severe neointima formation 8 weeks after transplantation (Figure 1A,B). RSG treatment resulted in a marked reduction in the surface neointima in aortic allografts (Figure 1C,D) as we showed previously.[27]

Rosiglitazone impairs PE-induced vasoconstriction

To assess whether abdominal aortic allografting with RSG treatment modulated the peripheral vascular response to a sympathetic stimulus, contraction in recipient thoracic aorta rings in response to phenylephrine (PE) was measured. As shown in Figure 2, aortic allografting without RSG treatment (open circles) did not change the contractive response to PE compared with age-matched non-treated, non-transplanted controls (open triangles). However, RSG treatment in transplanted rats induced a moderate but significant decrease in the maximal contractive response to PE ($p<0.05$ *vs.* non-treated but transplanted rats).

Rosiglitazone improves EC-dependent vasodilator function

To determine whether abdominal aortic allografting in rats causes SED, EC function was measured in thoracic aorta rings of transplanted and non-transplanted rats. To this end, EC-dependent relaxation of medial VSMC's was measured as the dilatory response to metacholine (ME). The net vasodilator response depends on the release of EC-derived relaxing factors and the responsiveness of medial VSMC's to these factors. As shown in Figure 3A, abdominal aortic allografting without further treatment (open circles) caused a more than 50% reduction of E_{max} of the EC-dependent vasodilation to cumulative dosages of ME, 8 weeks post-transplantation compared with age-matched non-treated, non-transplanted control rats (open triangles) (Figure 3A, $p<0.01$). RSG treatment of transplanted rats improved but did not normalize the net EC-dependent vasodilator response to cumulative dosages of ME (filled circles) (Figure 3A, $p<0.05$ *vs.* non-treated but transplanted rats). These data indicate that RSG has a beneficial effect on EC function. When expressing the

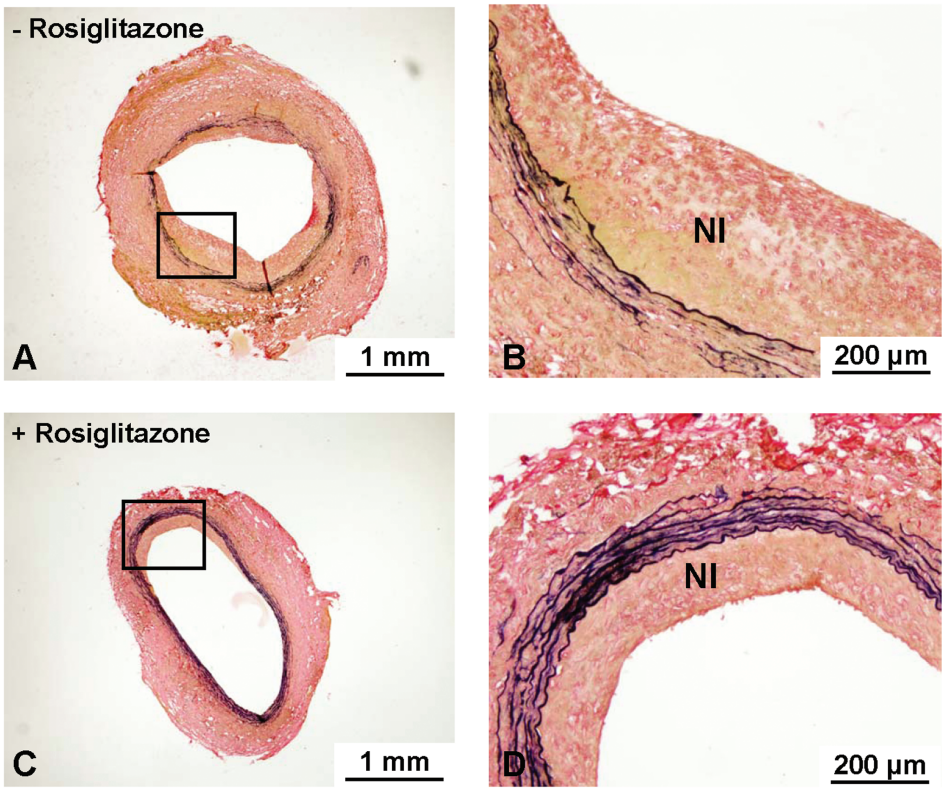

Figure 1. Rosiglitazone attenuates neointima formation in aortic allografts. BN recipient rats were treated with rosiglitazone or left untreated as described in *Methods*. (A) Transplant arteriosclerosis in non-treated recipients is characterized by disruption of the elastic laminae in the media and severe neointima formation. (B) Higher power magnification of the framed area shown in A (magnification x100). (C) Treatment with rosiglitazone resulted in reduced neointima formation compared with the non-treated control group. (D) Higher power magnification of the framed area shown in C (magnification x100). Staining performed on the sections shown in A-D: Verhoeff's elastin staining. Abbreviation: NI: neointima.

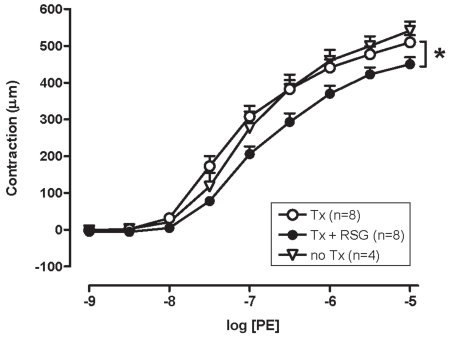

Figure 2. Rosiglitazone (RSG) treatment after transplantation reduces VSMC contractive responses to PE. Aortic allografting without RSG treatment (Tx, ○, n=8) does not change the contractive response of VSMC's to PE compared with non-treated, non-transplanted controls (no Tx, ▽, n=4). Treatment with RSG after transplantation (Tx + RSG, ○, n=8) significantly lowers (*, $p<0.05$) the maximal contractive response to PE compared with transplantation without RSG treatment (Tx, ○, n=8). (Data are presented as mean ± SEM, GLM for Repeated Measures).

dilatory response as a percentage of the maximal contractive response thereby correcting for the PE pre-contraction values, RSG was indeed shown to significantly improve EC-dependent vasodilation (filled circles) compared with non-treated but transplanted rats (open circles) (Figure 3B, $p<0.05$) to a level that was not statistically different from age-matched non-treated, non-transplanted controls (open triangles).

Rosiglitazone impairs VSMC vasodilator function

To determine the total (EC-dependent and independent) dilatory response in non-transplanted and transplanted rats (with or without RSG treatment), thoracic aortic rings were stimulated with sodium nitrite (SN) as an exogenous NO source. As shown in the left part of Figure 4A, aortic transplantation (white bar) resulted in a significantly reduced dilatory response to SN compared with age-matched non-treated, non-transplanted controls (grey bar, $p<0.001$). RSG treatment (black bar) further reduced the responses to SN ($p<0.01$ *vs.* non-treated but transplanted). This effect of RSG on the total dilatory response occurred independently of RSG-induced reduction of VSMC contractility (as shown in Figure 2) since similar effects were observed when expressing the total dilatory response as a percentage of pre-contraction values to PE (Figure 4A, right part).

Since the EC-dependent vasodilatory response was improved (as shown in Figure 3) but the total vasodilatory response to SN was impaired (as shown in Figure 4A) after RSG treatment, we corrected the ME responses for the response to SN. This corrected response determines the ability of the endothelium to release relaxing factors upon RSG treatment. ME responses corrected for the response to SN are shown in Figure 4B. Aortic allografting (open circles) resulted in a significantly reduced dilatory response ($p<0.01$ *vs.* age-matched

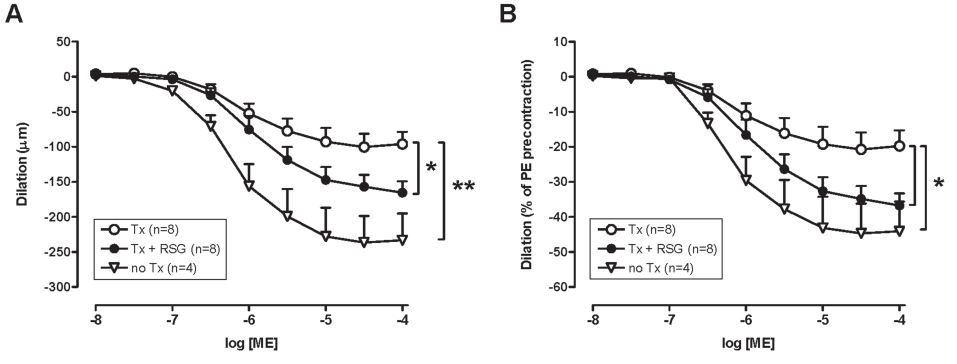

Figure 3. Rosiglitazone (RSG) improves EC-dependent vasodilator function after aorta transplantation. (A) Aortic allografting (Tx, ○, n=8) resulted in significantly reduced vasodilation in response to metacholine (ME) (as determined 8 weeks after transplantation) compared with age-matched non-treated, non-transplanted controls (no Tx, ▽, n=4) (**$p<0.01$). Treatment with RSG (Tx + RSG, ○, n=8) improved vasodilator function compared with non-treated, transplanted rats (Tx, ○, n=8) (* $p<0.05$) (B) When dilatory responses were expressed as a percentage of maximal contraction to phenylehprine (PE), similar results were obtained (* $p<0.05$). RSG treatment restored transplantation-induced systemic endothelial dysfunction to levels that did not statistically differ from age-matched non-treated, non-transplanted controls. (Data are presented as mean ± SEM, GLM for Repeated Measures).

non-treated, non-transplanted rats, open triangles) reflecting reduced release of relaxing factors by EC's after transplantation. The ability of EC's to release relaxing factors was fully restored after treatment with RSG (filled circles) ($p<0.01$ *vs.* non-treated but transplanted rats) even showing a trend towards improved EC function as compared to age-matched non-treated, non-transplanted controls (open triangles).

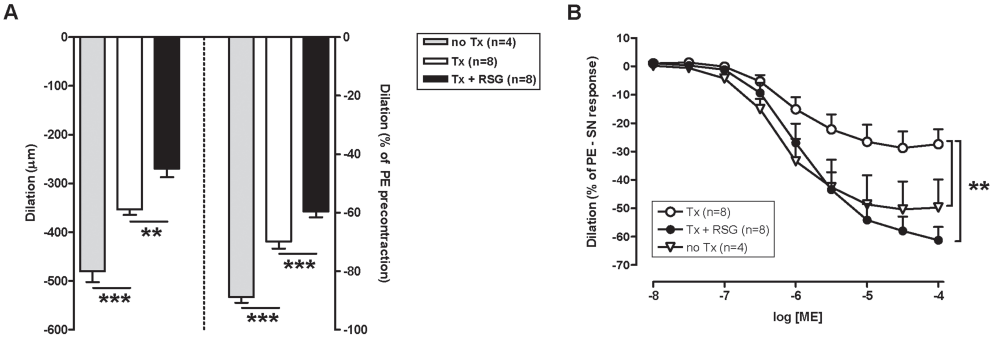

Figure 4. Rosiglitazone (RSG) reduces total dilatory responses to SN. (A) Aortic allografting results in reduced vasodilation (response to 10 mM sodium nitrite [SN]) compared with age-matched non-transplanted controls. RSG-treatment further reduced the dilatory response. Responses are expressed either in µm (left panel) or as a percentage of maximal contraction to phenylephrine (PE) (right panel). (Data are expressed as mean ± SEM, Students' t-test, ** $p<0.01$, *** $p<0.001$) (B) After correction of the ME responses for the responses to SN, data indicate that RSG treatment (Tx + RSG, ○, n=8) reestablishes the ability of endothelium to release relaxing factors compared with aortic allografting in non-treated rats (Tx, ○, n=8) (* $p<0.01$). (Data are presented as mean ± SEM, GLM for Repeated Measures).

Discussion

Development of CVD after transplantation is characterized by insulin-resistance and SED and is associated with significant morbidity and mortality.[3,5-8] Since PPARγ agonists like RSG have been shown to improve EC function in diabetes and hypertension[14-18] we tested the hypothesis that RSG also improves transplantation-induced SED in addition to its beneficial effect on the development of transplant arteriosclerosis.[27] Our data indicate that aorta transplantation induces SED which is restored to pre-transplantation levels after treatment with RSG. On the other hand, RSG induces VSMC dysfunction as demonstrated by a moderate reduction in VSMC contractility and a pronounced decrease in VSMC dilatory capacity.

After aortic transplantation in rats SED developed which was characterized by reduced EC-dependent vasodilation. Experimental and clinical data suggest a strong relation between inflammation and endothelial dysfunction.[39,40] Therefore, transplantation-induced SED in our model is most likely the result of a chronic systemic inflammatory state of the allo-condition which parallels our previous observations after experimental renal transplantation[9] and stenting[41] in rats.

RSG treatment after transplantation restored EC-dependent vasodilation which was most likely due to improved ability of EC's to release vasodilating factors. However, we observed that *in vivo* exposure to RSG induced a reduction in VSMC contraction in response to a sympathetic (PE) stimulus. More importantly, despite complete restoration of EC-dependent dilator function, the total dilatory response (that comprises an endothelium-dependent and an endothelium-independent VSMC response) was reduced. These data indicate that the favorable effect of RSG on EC function after transplantation is counteracted by direct EC-independent deleterious effects on VSMC dilatory potential, as witnessed by the reduced response to exogenous nitric oxide (response to sodium nitrite).

As anti-inflammatory treatment strategies are known to improve EC function[42,43], the anti-inflammatory properties of RSG[11] might have contributed to preservation of EC function after aortic transplantation. Alternatively, or together with EC preservation, RSG treatment might have facilitated endothelial repair mediated by endothelial progenitor cells (EPC's). EPC's are involved in maintaining endothelial integrity under stressful conditions.[44] The number of circulating EPC's correlates with EC-dependent vasodilation[45] and EPC-mediated repair of vascular injury is associated with normalization of endothelial function at the site of injury.[46] RSG has been shown to facilitate EPC differentiation and function[24,47] and to enhance EPC-mediated reendothelialization.[48] Presence of EPC-mediated systemic vascular repair after transplantation is currently under investigation.

In our study, RSG was shown to reduce the total dilatory response and, to a lesser extent, PE-induced VSMC contraction. The effect of RSG on EC-independent vasodilation tended to counteract the gain of endothelial function. In line with our data, RSG-induced reduction of VSMC contraction in response to sodium fluoride or thromboxane A_2 was recently described although in this study RSG did not affect PE-induced contraction.[49] These findings show that impaired contractility is not limited to adrenergic stimuli. Furthermore, RSG-induced reduction of VSMC contraction in response to PE in our transplantation model might reflect an adaptive response of VSMC's to their transplantation-induced reduction of dilator capacity.

Taking in consideration that EC function is effectively conserved by RSG after transplantation, the repressing effects on VSMC contraction and dilation stand out all the more. In addition to a relatively mild impairment of contractile potential, RSG treatment in transplant recipients leads to a pronounced decrease in VSMC dilator capacity. The mechanism remains to be explored, and might involve both morphological as well as pharmacological changes. Morphological changes such as deposition of extracellular matrix and VSMC hypertrophy might increase stiffness of the vessel wall and thus results in both decreased contractile as well as dilator function. The group of pharmacological changes comprises a large variety of putative mechanisms, including changes in receptor expression, second messenger activation (Ca^{2+} mobilization, cGMP production), decrease of nitric oxide (NO) function *e.g.* by changes in NO production or scavenging by reactive oxygen species, loss of actin and myosin function. Within the limitations of the current study our data clearly demonstrate that the pronounced effect of RSG on vasomotor function is not due to its impact on EC function but a matter of functional changes in VSMC's.

However, from our study no conclusions can be drawn on the mechanism(s) involved in RSG-induced VSMC dysfunction after transplantation and more comprehensive studies need to be performed to address this issue.

Given the pro-inflammatory and disturbed metabolic status (characterized by insulin resistance and SED) after clinical transplantation[5,8], transplanted subjects would benefit from a therapy that is immunosuppressive (to reduce rejection and transplant arteriosclerosis) but simultaneously improves insulin-sensitivity and EC function. Based on our previous results[27] and data reported in this study RSG may fulfill these criteria. Exposing patients eligible for transplantation to RSG should however be considered with caution, especially in the view of current debate on the effect of RSG on increasing cardiovascular risk in Type 2 diabetics.[32-36,50] Although our data clearly indicate that RSG restores transplantation-induced endothelial dysfunction, also a clear EC-independent deleterious effect on VSMC contraction and relaxation was observed. These dichotomous effects of RSG might impede use of RSG in the transplantation setting since this can potentially increase the risk for CVD despite improving EC function.

Acknowledgments

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6

Non-bone marrow origin of neointimal smooth muscle cells in experimental in-stent restenosis in rats

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Abstract

Objective: To determine the contribution of bone marrow (BM)-derived cells in in-stent restenosis (ISR) and transplant arteriosclerosis (TA).

Methods: Non-transgenic rats WT F344^{TG} (n = 3) received stent implantation 6 weeks after lethal total body irradiation and suppletion with bone marrow from a R26-hPAP transgenic rat. After 4 weeks the abdominal aortas were harvested, the stent was quickly removed, the abdominal aorta was snap-frozen in liquid nitrogen and 5 μm cryosections for stainings were cut. Additionally, DA aortic allografts were transplanted into WT F344^{TG} (n = 3) and R26-hPAP^{WT} (n = 3) BM-chimeric recipients. Immunohistochemistry (hPAP staining) and immunofluorescence (hPAP, α-SMA and OX1) was performed on all sections.

Results: Few hPAP-positive cells were observed in the neointima. Double stainings of hPAP-positive areas showed no α-SMA colocalization; OX-1 did show colocalization.

Conclusions: Non-BM-derived cells are the predominant source of neointimal cells in ISR and TA. Vascular wall-derived progenitor cells may rather be the source of SMCs that contribute to ISR and TA, which may have implications for our quest for new therapeutic targets to treat these vasculopathies.

Introduction

Development of in-stent restenosis (ISR) is the most common complication associated with coronary stenting, especially in patients treated with bare-metal stents. No adequate treatment modalities are available to treat or prevent development of ISR.[1,2] Recruitment and proliferation of smooth muscle cells (SMCs) in response to vascular injury after stenting are key phenomena that lead to the development of an occlusive neointima culminating in ISR.[3,4] Although it is well established that in humans the neointima of stented vessels is mainly composed of α-smooth muscle actin (SMA) positive cells[5], the origin of neointimal cells in ISR is still a matter of debate. Identifying the anatomical origin and molecular characteristics of the progenitor cells that ultimately form the neointima in ISR is of importance since these cells form a putative target for therapeutic intervention to prevent ISR and related occlusive vascular diseases like transplant arteriosclerosis (TA). Along with the classical theory of inward migration and proliferation of medial SMCs[6], a more recent proposed hypothesis attributes an important role to bone marrow (BM)-derived vascular progenitor cells in the process of neointima formation.[7] BM contains both hematopoietic and mesenchymal stem cells which have the capacity to self-renew and to differentiate into a variety of cell types including SMCs.[8,9] Given the potential of BM stem cells to give rise to SMCs, SMC progenitors might be recruited from the BM into the circulation in response to vascular injury and home to the site of injury resulting in neointima formation eventually. In line with this, various animal models of vascular injury (atherosclerosis, wire injury and TA) indeed demonstrate contribution of BM-derived cells in neointima formation to some extent.[10-13] However, results described so far are not conclusive since we and others demonstrated that in experimental TA and (vein graft) atherosclerosis neointimal SMCs are primarily non-BM-derived.[14-16] The contribution of BM-derived cells in the development of ISR is largely unknown although some recent studies suggest involvement of BM-derived cells based on increased numbers of circulating $CD34^+$ cells shortly after stenting[17] and the presence of neointimal cells expressing stem cell antigens like c-kit[18,19], CD34 and AC133.[20] However, although these markers are indeed expressed on primitive cells residing in the bone marrow, expression is not strictly confined to BM-derived cells. As a result, neointimal cells expressing these markers in ISR are not derived from the BM by definition. Direct evidence of involvement of BM-derived cells in the development of ISR has thus not been reported so far. Since the contribution of BM-derived cells in neointima formation is most likely dependent on the severity of endovascular injury[11,21], it is of importance that studies on the origin of neointimal cells in specifically ISR are performed in a relevant model of true ISR and not a model of otherwise induced endovascular injury. In this study we therefore determined the contribution of BM-derived cells in ISR in a direct way using our recently developed model of ISR[22] using genetically marked BM chimeric rats.

Methods

Rats

Male wild-type (WT) Fischer344 (F344) and Dark Agouti (DA) rats were obtained from Harlan Nederland (Horst, The Netherlands). Human Placental Alkaline Phosphatase (hPAP) transgenic F344 rats (R26-hPAP rats) were derived from a breeding nucleus provided by Dr. E.P. Sandgren (University of Wisconsin-Madison, USA).[23] Rats were kept under clean conventional conditions and were fed standard rat chow and acidified water *ad libitum*. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and the Dutch Law on Experimental Animal Care.

Bone marrow transplantation

Both femora and tibiae of BM donor rats were excised and surrounding muscle and connective tissue were removed and the BM was flushed with sterile PBS. Erythrocytes were lysed in lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM sodium ethylenediaminetetraacetic acid [EDTA]), and the cell suspension was then filtered through a 20 μm cell strainer (Becton Dickinson, Alphen aan den Rijn, The Netherlands). BM recipient rats were lethally γ-iradiated (9 Gy) using a ^{137}Cesium source (IBL 637, CIS Bio International). One hour after irradiation rats were reconstituted with 1-5x10^7 BM cells by tail vein injection. Three experimental groups were included: 1) hPAP Tg F344 BM -> WT F344 [WT F344^{TG}], stented 6 wks after reconstitution; 2) hPAP Tg F344 BM -> WT F344 [WT F344^{TG}], aorta allografted 6 wks after reconstitution; 3) WT F344 BM -> hPAP Tg F344 [R26-hPAP^{WT}], aorta allografted 6 wks after reconstitution. BM chimeric rats were housed in filtertop cages throughout the duration of the experiment. Rats received drinking water containing neomycin (0.35% wt/vol) starting 1 week before irradiation until 2 weeks after BM reconstitution. Six weeks after BM reconstitution and prior to stenting or allografting the level of chimerism was determined by flow cytometric analysis on peripheral blood mononuclear cells (PBMNCs). The level of chimerism was typically between 80% and 90% (data not shown).

Stent implantation

Chimeric rats (6 wks after BM reconstitution) received a stent in the abdominal aorta as described in detail elsewhere.[22] Briefly, under anesthesia (2% isoflurane [Abbot, Hoofddorp, The Netherlands], 0.4 L/min O_2 and 0.4 L/min N_2O) the abdominal cavity was opened. The aorta was dissected and surrounding connective tissue was removed. Next, two vascular clips were placed onto the aorta distal to the renal arteries and proximal to the aortic bifurcation. A small incision was then made in the distal abdominal aorta and the balloon catheter was inserted and inflated to 9 atm pressure to deploy a pre-mounted 2.5 x 8 mm Micro-Driver stent (Medtronic, Minneapolis, United States, n=3). After deflation and removal of the balloon, the aortic incision was closed with a 9-0 suture. Reperfusion was established by removing the clips and the abdomen was closed with 4-0 sutures. Four weeks after surgery the stented aortas were harvested and the stents were carefully removed from

the lumen of the aorta. Aortic tissue was snap-frozen in liquid nitrogen and stored at -80°C for cryostat sections.

Aorta transplantation

Since we previously showed non-BM origin of neointimal vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) in transplant arteriosclerosis (TA) using allogeneic BM-chimeric rats[15,21], we also performed aortic allografting in WT F344^{TG} and R26-hPAP^{WT} BM-chimeric rats to test the feasibility of detecting (non)-BM-derived VSMCs and ECs in established neointimal lesions in the hPAP-transgenic F344 rat model. So far, this rat model has not been used to track (non)-BM-derived VSMCs and ECs in neointimal lesions. Under anesthesia (as described above) DA aortic allografts were transplanted into WT F344^{TG}(n=3) and R26-hPAP^{WT}(n=3) BM-chimeric recipients as described previously.[24] Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor, perfused with saline and subsequently orthotopically transplanted into the recipient via end-to-end anastomosis with total cold and warm ischemic time consistently less than 25 minutes.

Immunohistochemistry

To localize BM-derived hPAP-positive cells in ISR, an indirect immunoperoxidase staining for hPAP was performed on cryosections cut from the stented area. Sections (5 µm) were acetone-fixed (10 min., 4°C). Blockade of endogenous peroxidase (incubation 30 min. with PBS containing 0.03% H_2O_2) was followed by incubation for 60 min at room temperature with the primary polyclonal antibody against hPAP (AHP537HT, AbD Serotec, BioConnect, Huissen, The Netherlands) diluted in 1% BSA/PBS. Subsequently, the sections were incubated with a second-step horseradish peroxidase-conjugated goat-anti-rabbit antibody (DAKO A/S, Glostrup, Denmark) for 30 min diluted in 1% BSA/PBS supplemented with 1% normal rat serum. Peroxidase activity was developed using chromogen 3-amino-9-ethyl carbazole (AEC; DAKO A/S). Sections were counterstained with hematoxylin and mounted in Faramount (DAKO A/S). Control slides, in which the primary antibody was replaced with PBS were consistently negative (not shown).

Immunofluorescence

To further phenotype hPAP$^+$ cells in ISR triple-immunofluorescent staining was performed using anti-hPAP, α-SMA (SMCs; clone 1A4, mIgG2a, Dako A/S) and anti-CD45 (clone OX-1, mIgG1 tissue culture supernatant). Sections were incubated for 1 hr with a mixture of the primary antibodies (diluted in 1% BSA/PBS) followed by incubation with Alexa488-conjugated goat anti-mouse IgG2a (Molecular Probes, Leiden, The Netherlands), Cy5-conjugated goat anti-mouse IgG1 (Molecular Probes) and horseradish peroxidase-conjugated swine-anti-rabbit Ig (Dako A/S) in 1% BSA/PBS supplemented with 1% normal rat serum for 30 min. Horseradish peroxidase-conjugated swine-anti-rabbit Ig was detected using the TSA™ Tetramethylrhodamine System (PerkinElmer LAS, Inc., Boston, MA, USA). Nuclei were stained with DAPI and sections were embedded in Citifluor (AF1, Agar Scientific Ltd.,

Stansted, UK). To validate this four-color immunofluorescent staining protocol and check for potential crossreactivity of the isotype-specific second-step antibodies, first single stainings for hPAP, SMA and CD45 were performed on hPAP-transgenic F344 spleen sections.

Following a similar immunofluorescence protocol double staining for hPAP (polyclonal rabbit Ig) and α-SMA (mouse IgG2a), and hPAP and RECA-1 (mouse IgG1, endothelium)[25] were performed on 5 µm aortic graft cryosections. Binding of anti-hPAP antibodies was detected using FITC-conjugated goat anti-rabbit Ig (Dako A/S) whereas binding of α-SMA and RECA-1 antibodies was detected using horseradish peroxidase-conjugated rabbit-anti-mouse Ig (Dako A/S) which was visualized using the TSA Tetramethylrhodamine System. All fluorescently labeled sections were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems Nederland B.V., Rijswijk, The Netherlands).

Results

Specificity α-hPAP staining

Since the hPAP-transgenic F344 rat model has not been used before to track (non)-BM-derived VSMCs and ECs in neointimal lesions we first analyzed the specificity and sensitivity of our staining method to detect hPAP-transgenic BM and vascular wall cells. As shown in Figure 1, both BM cells (C) and medial VSMCs and ECs in non-injured aorta (D) from hPAP-transgenic F344 rats stained positive for hPAP using an hPAP-specific polyclonal antibody. For comparison, BM cells (A) and aortic tissue (B) from wildtype F344 rats did

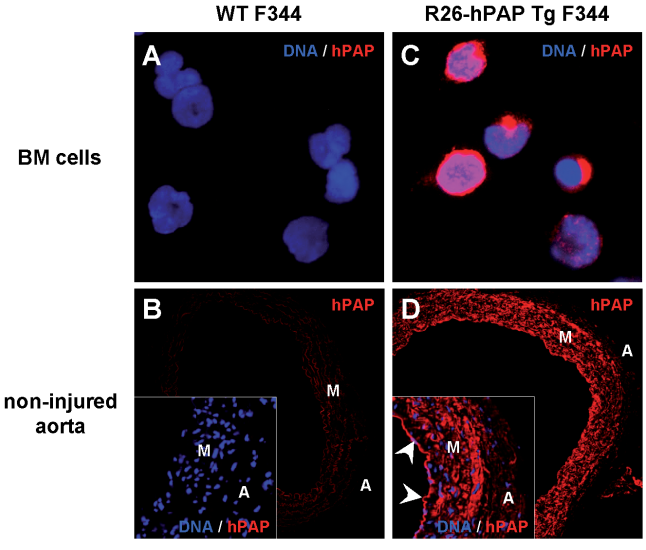

Figure 1. The α-hPAP staining is sufficiently sensitive and specific to detect hPAP-transgenic BM and vascular wall cells. BM cells (A) and aortic tissue (B) from wildtype F344 rats did not react with the α-hPAP antibody, whereas BM cells (C) and medial VSMCs and ECs (D; arrowheads inset) in non-injured aorta from hPAP-transgenic F344 rats clearly reacted with the α-hPAP antibody. A = adventitia; M = media. Magnification x1890 (A,C) and (B, D; inset magnification x630).

not react with the α-hPAP antibody. These results indicate that this staining method is specific and sufficiently sensitive to detect hPAP-expressing BM and vascular cells.

Development of 4-parameter (CD45, SMA, hPAP, DNA) immunofluorescent staining protocol

To validate a 4-parameter immunofluorescent staining protocol and check for potential crossreactivity of the isotype-specific second-step antibodies, single and triple stainings for hPAP, SMA and CD45 were performed on hPAP-transgenic F344 spleen sections. Sections were incubated with one primary antibody and then detected with a cocktail of 3 fluorochrome-labeled isotype-specific second-step antibodies. As shown in Figure 2, mIgG1 α-CD45 was only detected with α-mIgG1-Cy5 (A-E), mIgG2a α-SMA was only

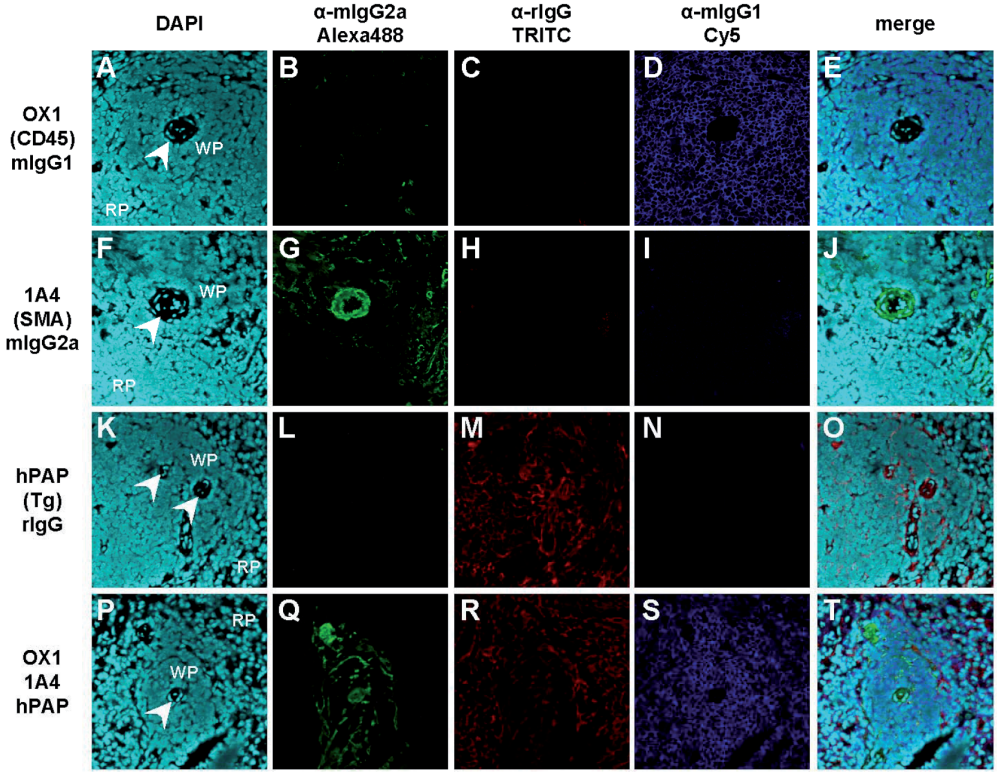

Figure 2. Simultaneous detection of CD45, SMA, hPAP and DNA by immunofluorescent staining on hPAP-transgenic F344 rat spleen. (A-E) Primary incubation with OX1 (α-CD45, mIgG1) and secondary incubation with α-mIgG1-Cy5, α-mIgG2a-Alexa488, and α-rIgG TRITC. OX1 reacted with the lymphocytes present in the white pulp (WP) and red pulp (RP) around the central arteriole (arrowhead) and was only detected with α-mIgG1-Cy5. (F-J) Primary incubation with 1A4 (α-SMA, mIgG2a) and secondary incubation with α-mIgG1-Cy5, α-mIgG2a-Alexa488, and α-rIgG TRITC. 1A4 reacted with the stromal cells present in the WP and medial VSMCs in the central arteriole (arrowhead) and was only detected with α-mIgG2a-Alexa488. (K-O) Primary incubation with α-hPAP (rIgG) and secondary incubation with α-mIgG1-Cy5, α-mIgG2a-Alexa488, and α-rIgG TRITC. α-hPAP reacted with all cells present in the WP and RP and was only detected with α-rIgG-TRITC. (P-T) Primary incubation with OX1, 1A4 and α-hPAP and secondary incubation with α-mIgG1-Cy5, α-mIgG2a-Alexa488, and α-rIgG TRITC. Expression of all antigens could be demonstrated simultaneously. Magnification x200.

detected with α-mIgG2a-Alexa488 (F-J), and rIgG α-hPAP was only detected with α-rIgG TRITC (K-O). When incubating sections with a mixture of CD45, hPAP and SMA primary antibodies, expression of all antigens could be demonstrated simultaneously (P-T). This 4-parameter immunofluorescent staining protocol was then used to determine the origin of neointimal VSMCs and ECs in TA and ISR.

Neointimal VSMCs and ECs in TA are non-BM-derived

Since we previously showed non-BM origin of neointimal VSMCs and ECs in TA using allogeneic BM-chimeric rats[15,21], we first performed aortic allografting in WT F344^{TG} and R26-hPAP^{WT} BM-chimeric rats to test our model system for specificity and sensitivity of detecting (non)-BM-derived VSMCs and ECs in established neointimal lesions. Two months after allografting both the WT-F344^{TG} (Figure 3A and B) and R26-hPAP^{WT} (Figure 3C and D) had developed marked TA characterized by a neointima consisting of a packed layer of SMA^{+} cells covered by ECs at the luminal side. The neointimal cells in allografts

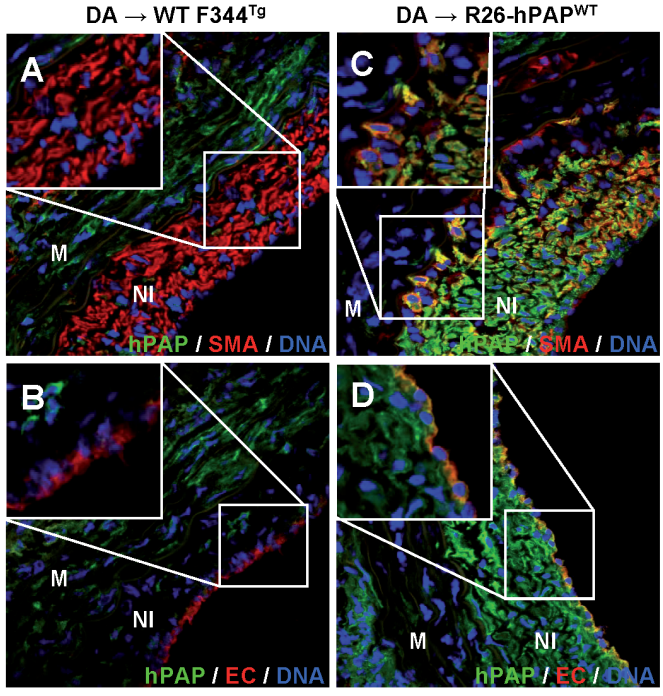

Figure 3. Neointimal VSMCs and ECs in TA are non-BM-derived. DA aortic allografts were transplanted in WT F344^{TG} (hPAP BM chimeric F344 wild-type rats; A, B) and R26-hPAP^{WT} (wild-type BM chimeric R26-hPAP transgenic rats; C, D) recipients and analyzed 2 months after transplantation. (A) Neointimal SMA^{+} VSMCs (red) do not express hPAP. Magnification x630. (B) RECA-1^{+} ECs (red) do not express hPAP. Magnification x630. Insets show high-power magnifications of neointimal VSMCs (A; magnification x1890) and ECs (B; magnification x2520) that do not express hPAP. (C) Colocalized expression of SMA (red) and hPAP (green) in neointimal VSMCs. Magnification x630. (D) Colocalized expression of RECA-1 (red) and hPAP (green) in neointimal ECs. Magnification x630. Insets show high-power magnifications of hPAP^{+} neonitimal VSMCs (C; magnification x1890) and ECs (D; magnification x2520). M = media; NI = neointima.

transplanted in wild-type recipients reconstituted with hPAP-transgenic BM (WT-F344^{TG}) expressed SMA but colocalization with hPAP-expression was not observed (0% BM-derived α-SMA$^+$ VSMCs, Figure 3A). Also neointimal ECs did not express hPAP (0% BM-derived RECA-1$^+$ VSMCs, Figure 3B). The BM-derived hPAP$^+$ cells that were detected in the neointima, media and adventitia expressed CD45, indicating that these cells were infiltrating leukocytes (data not shown). These results suggest a non-BM origin of the neointimal ECs and VSMCs in established TA. Analyses performed on allografts transplanted in hPAP-transgenic recipients reconstituted with WT BM (R26-F344^{WT}) confirmed this premise as shown in Figure 3C and D. Virtually all neointimal SMA$^+$ (Figure 3C) and ECs (Figure 3D) coexpressed the hPAP transgene, indicating ~100% non-BM origin of these cells in TA. These data confirm our previous observations and indicate that the hPAP-transgenic F344 rat model is sufficiently specific and sensitive to detect (non)-BM-derived VSMCs and ECs in established neointimal lesions.

Presence of BM-derived hPAP$^+$ cells in ISR

Stenting of the BM-chimeric rats resulted in the development of extensive and maximal ISR after 4 wks. In this model of ISR at earlier time-points only mild lesions (without

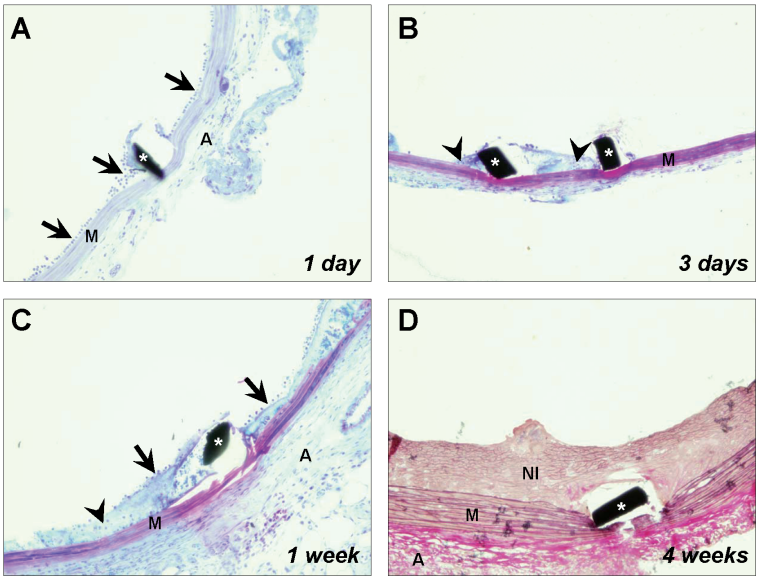

Figure 4. Kinetics of the development of ISR after experimental stenting in rats. (A) 1 day after stenting: local thrombus formation around the stent struts (asterisk) with surface adhering leucocytes (arrows). Toluidine blue staining. (B) 3 days after stenting: local thrombus formation around the stent struts (asterisks) with an increased number of infiltrating leucocytes (arrowheads). Toluidine blue-basic fuchsin staining. (C) 1 week after stenting: organized thrombus with surface-adherent leucocytes (arrows) and increased leucocyte infiltration (arrowhead). Toluidine blue-basic fuchsin staining. (D) 4 weeks after stenting: stent struts (asterisk) are completely covered by neointima which mainly consist of VSMCs and extracellular matrix with the absence of large numbers of infiltrating leucocytes. Elastica van Gieson staining. Magnification x200. A = Adventitia, M = media; NI = neointima.

SMA-positive VSMCs) are present which are characterized by local thrombus formation around the stent struts with surface adhering leucocytes (1 day) or thrombus-infiltrating leucocytes (3 days and 1 week). Figure 4 shows representative photomicrographs of the histological appearance of the composition of the lesions in developing ISR at 1 (A) and 3 days (B) and 1 (C) and 4 (D) weeks after stenting. Since the aim of this study is to determine the (non)-BM origin of neointimal VSMCs in established ISR, stented aorta's were analyzed 4 weeks after stenting.

After removal of the stents the neointima was still attached to the luminal side of the aortic wall (Figure 5). Immunohistochemistry for hPAP-transgene expression revealed the abundant presence of hPAP$^+$ cells in the adventitia (Figure 5A) and media (Figure 5B and C) whereas the neointima contained a relatively low number of hPAP$^+$ cells (Figure 5B and C).

Neointimal VSMCs in ISR are non-BM-derived

To determine the smooth-muscle-like phenotype of the BM-derived neointimal hPAP$^+$ cells in ISR, triple staining for hPAP, SMA and CD45 was performed. The neointima consisted primarily of SMA$^+$ VSMCs whereas the media was devoid of SMA$^+$ VSMCs after stenting (Figure 6B). Although the neointima contained considerable numbers of BM-derived hPAP$^+$ cells (Figure 6C) the absence of colocalization of hPAP and SMA expression was consistently observed in all animals analyzed (Figure 6E) indicating a non-BM origin of neointimal VSMCs in ISR (0% BM-derived SMA$^+$ VSMCs). Similar results were obtained for the neointimal ECs which were, however, sparse due to the mechanical removal of the

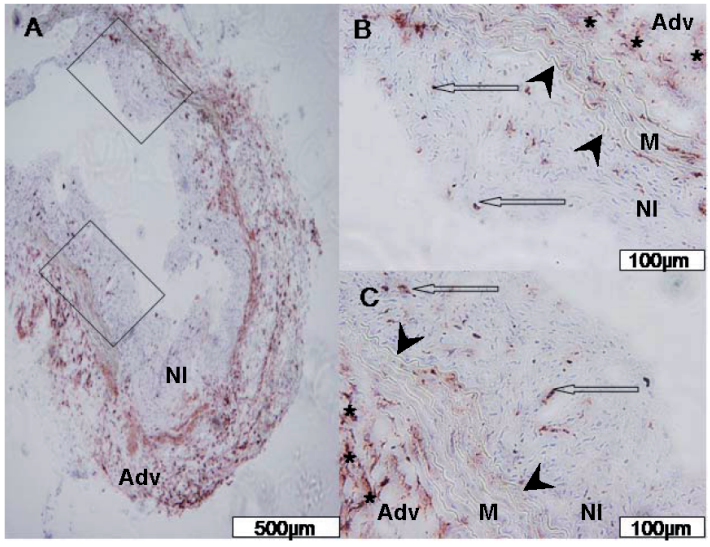

Figure 5. BM-derived hPAP$^+$ cells are present in ISR. Stenting was performed in hPAP-transgenic BM chimeric rats and analyzed 4 wks after stenting. Photomicrographs of neointima formed in stented aorta immunostained for hPAP and counterstained with hematoxylin. Few BM-derived hPAP$^+$ cells are present in the neointima (B, C; arrows), whereas hPAP$^+$ cells are abundantly present in the adventitia (B, C; asterisks). Arrowheads indicate the internal elastic lamina. Magnification x20 (A) and x200 (B, C) Adv = adventitia; M = media; NI = neointima.

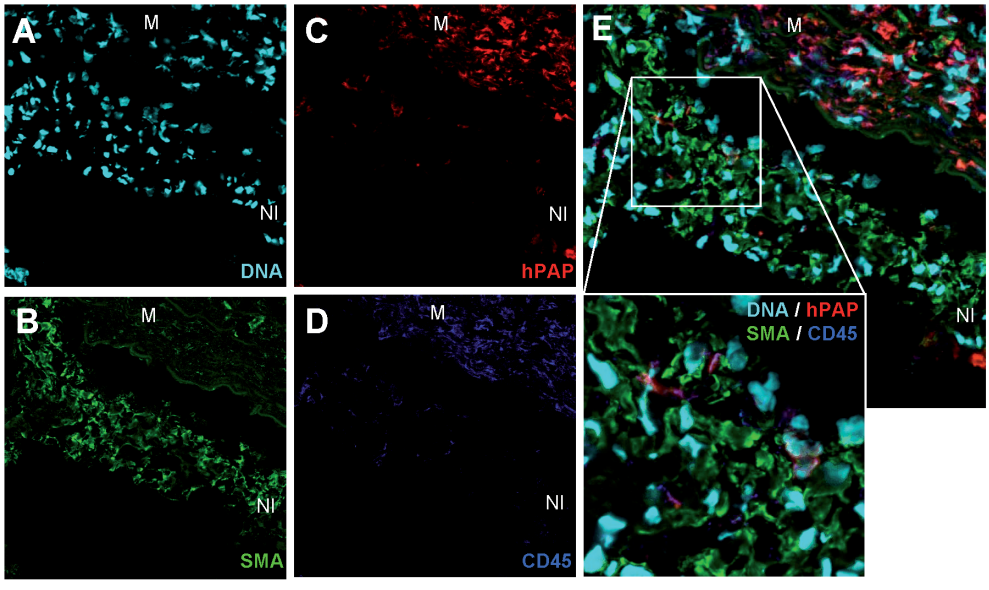

Figure 6. Neointimal BM-derived cells in ISR represent inflammatory cells but not VSMCs. Triple immunofluorescence staining for nuclear staining (A), SMA (VSMCs; B), hPAP (BM-derived cells; C) and CD45 (leukocyte common antigen, inflammatory cells; D). (E) Merged image of A-D showing no colocalization of hPAP and SMA expression and colocalization of hPAP and CD45 expression. Magnification x630. Inset shows high-power magnification of $hPAP^{-}$ neointimal VSMCs. Magnification x1890. M = media; NI = neointima.

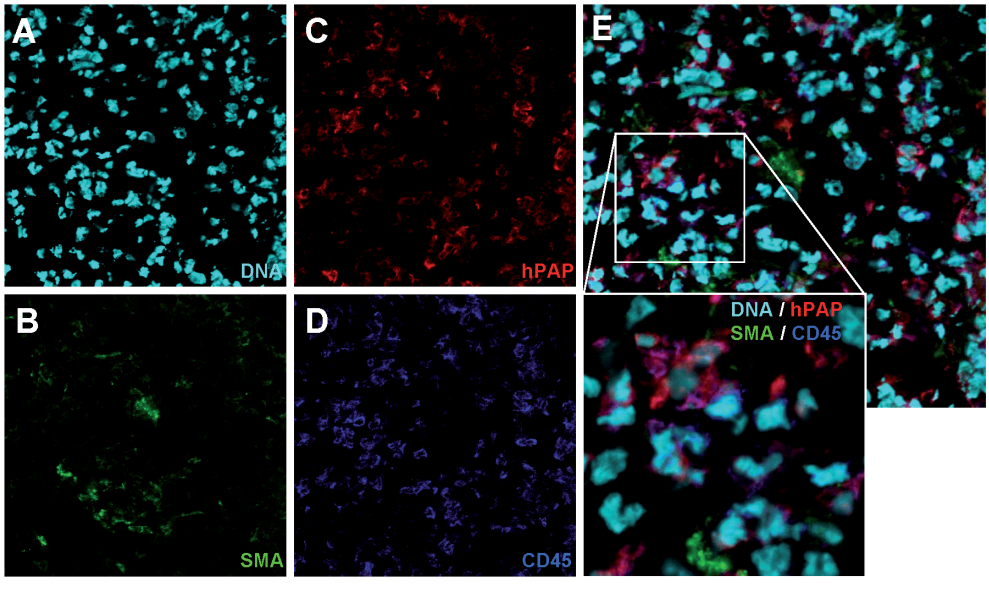

Figure 7. Adventitial BM-derived cells in ISR represent inflammatory cells. Triple immunofluorescence staining for nuclear staining (A), SMA (VSMCs; B), hPAP (BM-derived cells; C) and CD45 (leukocyte common antigen, inflammatory cells; D). (E) Merged image of A-D showing colocalization of hPAP and CD45 expression. Magnification x630. Inset shows high-power magnification of $hPAP^{+}$ inflammatory cells. Magnification x1890.

stents (data not shown). Colocalization of hPAP and CD45 expression in the neointima, media (Figure 6D and E) and adventitia (Figure 7) indicate that the BM-derived hPAP$^+$ cells in ISR were infiltrating leukocytes.

Discussion

In the present study in rats we determined the contribution of BM-derived cells in the development of ISR and TA after experimental stenting and aortic transplantation, respectively. In both models no BM-derived neointimal SMCs and ECs were detected and the few neointimal hPAP$^+$ BM-derived cells turned out to be CD45$^+$ infiltrating leukocytes. We conclude that vascular cells originating from the BM are not part of established neointimal lesions in both TA and ISR. Although the origin of neointimal cells has gained considerable interest in the last decade, only a few studies have been reported on the origin of neointimal SMCs after stenting.[17-20] Identification of the anatomical origin of the cells involved in development of ISR is of clinical importance since this may elucidate new targets that can be used for therapeutic interventions aimed at preventing or reducing such restenosis.

A putative source is the bone marrow. It is generally accepted that the BM contains hematopoietic and mesenchymal stem cells which can self-renew and differentiate into a variety of cell types including SMCs.[8,9] Furthermore, the human peripheral blood contains CD34$^+$ SMC progenitors[26] and, therefore, the BM is a putative source of SMCs involved in the development of ISR. In line with this, increased frequencies of circulating CD34$^+$ cells were detected after coronary stenting[17] and which was found to correlate with the late lumen loss (*i.e.* ISR) in stented patients.[27] It is not only in numerical difference that progenitor cells appear to correlate with the development of ISR, they also do so in their differentiation fate.[17] *In vitro*, mononuclear cells isolated from patients with ISR preferentially differentiated into α-SMA$^+$ while those from patients without ISR became endothelial-like cells. This indicates that differentiation in favor of SMCs may predispose for ISR.[17] Despite these correlative studies, direct evidence of involvement of BM-derived cells in the development of ISR has not been reported. Furthermore, in clinical ISR it is hard to discriminate between potential BM-derived cells that appeared after stenting and those that were already present in the vicinity of the stenotic area before stenting.[17,20]

In our model[22], no atherosclerosis is present at the time of stenting, which allows analysis of the direct effect of stenting on the recruitment of BM-derived cells and the development of ISR as reported in this article. However, in human atherosclerosis it has been shown that about 10% of the intimal cells in atherosclerotic plaques are derived from the BM.[13] If these BM-derived cells are a main source for the SMA$^+$ VSMCs in ISR after stenting of the atherosclerotic lesion, pre-existing atherosclerosis might result in a higher percentage of BM-derived VSMCs in ISR than observed in our study without the presence of pre-existing atherosclerosis. Recently, cells expressing stem cell antigens like CD34, c-kit[18-20] and AC133 have been shown to be present in ISR albeit at low levels (maximal ~11%). Taking into account the indirect way of detecting putative BM-derived cells in these studies, our data are,

in fact, quite similar and support the previously published data that the BM compartment is only marginally involved in the development of established ISR, if it is involved at all. Although the BM has been shown to harbor the potential to provide cells that contribute to neointima formation in various models for vascular injury other than ISR, the actual contribution of these cells is relatively low.[11,12,28] Indeed, we and others have showen that a non-BM source predominantly provides the cells involved in neointima formation in restenosis and TA.[11,14,15] A potential explanation for the differences in the contribution of BM-derived cells between previous reports[11,12,28] and the current study is the severity of vascular injury, since BM contribution in neointima formation appears to be dependent on the severity of endovascular injury.[11,21] However, we believe that our experimental model of ISR in rats also produces solid mechanical endovascular injury as measured by the injury scores as reported previously[22], which makes differences in severity of endovascular injury a less likely explanation for the observed differences in BM contribution.

In this study we for the first time, clearly demonstrate that in experimental ISR in rats the neointimal VSMCs are derived from a non-BM source. The BM thus plays a minor role in the development of established ISR. However, our results do not exclude the possibility that soon after stenting BM-derived cells are recruited to the injured vascular wall and create a microenvironment in which local progenitor cell niches are activated and mobilized by BM-derived cells in a paracrine fashion. Localized progenitor cell niches in the media[29] and the adventitia[30,31] of the vascular wall have been recently identified. Furthermore, isolated adventitial Sca-1$^+$ progenitor cells were shown to differentiate into VSMCs *in vitro*, and also to contribute to the development of atherosclerotic lesions *in vivo*.[30] The contribution of vascular wall-derived progenitor cells in the development of ISR and TA is as yet unknown but is currently under investigation.

In conclusion, non-BM-derived cells are the predominant source of neointimal cells in ISR and TA. Vascular wall-derived progenitor cells may be rather the source of SMCs that contribute to ISR and TA which may have implications for our quest for new therapeutic targets to treat these vasculopathies.

Acknowledgments

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7

Long-term type 1 diabetes enhances in-stent restenosis after aortic stenting in diabetes prone BB rats

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Abstract

Type 1 diabetic patients have increased risk of developing in-stent restenosis following endovascular stenting. Underlying pathogenetic mechanisms are not fully understood partly due to the lack of a relevant animal model to study the effect(s) of long-term autoimmune diabetes on development of in-stent restenosis. We here describe the development of in-stent restenosis in long-term (~7 months) spontaneously diabetic and age-matched, thymectomized, non-diabetic Diabetes Prone BioBreeding (BBDP) rats (n=6-7 in each group). Diabetes was suboptimally treated with insulin and was characterized by significant hyperglycaemia, polyuria, proteinuria, and increased HbA_{1c} levels. Stented abdominal aortas were harvested 28 days after stenting. Computerized morphometric analysis revealed significantly increased neointima formation in long-term diabetic rats compared with non-diabetic controls. In conclusion, long-term autoimmune diabetes in BBDP rats enhances in-stent restenosis. This model can be used to study the underlying pathogenetic mechanisms of diabetes-enhanced in-stent restenosis as well as to test new therapeutic modalities.

Introduction

In-stent restenosis (ISR) is the most common complication associated with coronary stenting and is histologically characterized by occlusive neointima formation. As yet, no adequate treatment modalities are available to treat or prevent development of ISR.[1-3] The use of drug-eluting stents has significantly reduced the incidence of ISR when compared with bare metal stents, but also resulted in increased rates of late stent thrombosis.[4] Diabetes mellitus (DM) has been associated with increased risk to develop (in-stent) restenosis both after percutaneous transluminal coronary angioplasty as well as coronary stenting.[5-14] DM is a risk factor for ISR development after using both drug-eluting and bare metal stents as revealed by various meta-analyses (primarily including Type 2 diabetic patients).[15-17] The beneficial effects on ISR of drug-eluting stents over bare metal stents as observed in non-diabetic patients appears to be less clear in diabetic patients. Although the exact pathogenetic mechanism underlying increased ISR development in diabetic patients is as yet unknown, it at least appears to be due to an exaggerated neointimal response after coronary-stent placement as determined by intravascular ultrasound.[18] Many studies thus favor for a deleterious effect of DM on the development of ISR, although the precise magnitude of this effect is not clear. Especially age may act as a confounder as older age is associated with increased restenosis rates independent of DM.[17]

To study the molecular and cellular mechanism(s) underlying DM-enhanced development of ISR, clinically relevant animal models may be of great value. However, despite the increasing numbers of diabetic patients worldwide together with coronary artery disease being a significant source of morbidity and mortality in these patients, relevant animal models to study ISR are scarce. Although the Zucker diabetic fatty (ZDF) rat has been used as a model to study Type 2 DM-associated restenosis[19-21], a reliable model to study the long-term effects of Type 1 diabetes on ISR development is still lacking. Whereas Type 1 DM represents only 5-10% of all diabetics it may be associated with severe coronary artery disease at a relatively young age as demonstrated in a selected population of Type 1 diabetics eligible for kidney and/or pancreas transplantation.[22] Various rodent models of Type 1 DM have been used to study the effects of diabetes on mechanically-induced restenosis in mice, rats or rabbits, in which diabetes is chemically induced using streptozotocin or alloxan and in which contradicting results were obtained.[23] In addition to rodent models, also a streptozotocin-induced diabetes porcine model has been used to study the development of ISR. In this model, a high mortality rate (~45%), a relatively short course of diabetes (12 weeks) and most likely high costs may actually hamper extensive use of this model.[24] Both streptozotocin and alloxan are toxic compounds sharing structural similarities with glucose which explains their selective uptake in cells expressing the GLUT2 glucose transporter. As pancreatic ß-cells have relatively high levels of GLUT2, streptozotocin and alloxan are relatively, but not solely, toxic to ß-cells. Because of the possible toxic side effects of these chemicals and to model more accurately human Type 1 (autoimmune) DM, the availability of a spontaneous Type 1 DM model for the development of (in-stent) restenosis is needed. To this end, we here describe the use of inbred Diabetes Prone BioBreeding (BBDP/Wor) rats

as a model to study diabetes-enhanced development of ISR. Inbred BBDP rats are derived from a Canadian colony of outbred Wistar rats (*i.e.* the BB Wistar rat) in which diabetes developed spontaneously in the 1970s.[25] BBDP/Wor rats develop autoimmune diabetes spontaneously due to severe lymphopenia and preferential lack of immunoregulatory T cells.[26-28] Using the rat abdominal aorta stenting model described previously[29], we tested the hypothesis that long-term suboptimally-treated hyperglycaemic BBDP rats develop enhanced ISR compared with non-diabetic age-matched control rats.

Materials and methods

Rats

Specified pathogen free Diabetes Prone BB (BBDP/Wor) and Diabetes Resistant (BBDR/Wor) rats were bred at the Central Animal Facility of the University Medical Center Groningen, University of Groningen, Groningen, The Netherlands. Original breeding stocks were obtained from Biomedical Research Models (BRM Inc, Worcester, MA, USA). Rats were kept under clean conventional conditions and were fed standard rat chow and acidified water *ad libitum*. All animals received humane care in compliance with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1996) and the Dutch Law on Experimental Animal Care.

Diabetes development in BBDP rats

In the BBDP/Wor colony maintained at the University Medical Center Groningen, 80-90% of the rats spontaneously develop diabetes from ~70 days of age. Rats were considered diabetic when blood glucose levels exceeded 15 mmol/L as measured in peripheral blood obtained by tail vein puncture and by using a glucose sensor (Accu-Chek Sensor Comfort, Roche Diagnostics Nederland B.V., Almere, The Netherlands). In this study, 9 recent onset diabetic BBDP rats were initially included (both male and female) which received a ½ (~3-4 mm length) Linplant® sustained release insulin implant (©LinShin Canada Inc, Toronto, ON, Canada) subcutaneously at diagnosis using a trocar. According to the manufacturer the implants had an estimated insulin release of ~1 U/24 h/(½) implant for >40 days. We aimed at maintaining the blood glucose values in insulin-treated diabetic rats between 15-20 mmol/L during the entire follow-up period (up to 10 months of age). During follow-up diabetic rats were weighed 2-3 times a week. In case of weight loss, blood glucose levels were measured as described above. When blood glucose levels exceeded 20 mmol/L or blood glucose levels were between 15-20 mmol/L in the presence of substantial weight loss, rats were reimplanted with an insulin implant as described above.

In addition to the insulin-treated diabetic BBDP rats also a control group of age-matched non-diabetic BBDP rats (initially n=9) was included. Diabetes development was prevented by performing thymectomy at the age of 21 days as we described in detail elsewhere.[30] Weight and blood glucose levels of thymectomized non-diabetic BBDP rats were measured once every two weeks to obtain basal values.

HbA_{1c} measurements

To determine whether long-term suboptimal insulin treatment of diabetic BBDP rats is associated with increased glycated hemoglobin (HbA_{1c}) levels indicative of poor glycaemic control, we analyzed HbA_{1c} levels in a separate cohort of long-term diabetic BBDP rats (n=5), as well as in spontaneously protected non-diabetic BBDP rats (n=2) and non-diabetic BBDR/Wor rats (n= 6). HbA_{1c} levels were determined in freshly obtained peripheral blood (tail vein puncture) using the BIO-RAD in2it™ A1C analyzer (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Values are expressed as DCCT- HbA_{1c} %.

Renal function measurements

Two weeks before stenting renal function of the long-term diabetic and non-diabetic BBDP rats was determined by housing the rats in individual, urine-collecting metabolic cages for 24 hours and by collection of blood plasma. Plasma and urine creatinine levels were determined using the enzymatic colorimetric assay CREA plus (Roche Diagnostics GmbH, Mannheim, Germany).[31] Total urinary protein excretion was determined using the Roche Diagnostics Urinary/CSF Protein assay (Roche Diagnostics GmbH, Mannheim, Germany).[32,33]

Stent implantation

At a median diabetes duration of 28 weeks (7 months) 9 BBDP rats were stented in the abdominal aorta as described previously.[29] As a control, 9 age-matched, thymectomized non-diabetic BBDP rats were included. Briefly, under anesthesia [2% isoflurane (Abbott, Hoofddorp, The Netherlands), 0.4 L/min O_2 and 0.4 L/min N_2O] the abdominal cavity was opened. The aorta was dissected and surrounding connective tissue was removed. Next, two vascular clips were placed onto the aorta distal to the renal arteries and proximal to the aortic bifurcation. A small incision was then made in the distal abdominal aorta and the balloon catheter was inserted and inflated to 9 atm pressure to deploy a pre-mounted 2.5 x 9 mm BeStent^{tm} 2 bare metal stent (Medtronic-Bakken Research, Maastricht, The Netherlands). After deflation and removal of the balloon, the aortic incision was closed with a 9-0 suture. Reperfusion was established by removing the clips and the abdomen was closed with 4-0 sutures. Starting 5 days before stenting until the end of the experiment, rats were fed chow containing 0.33 mg clopidogrel/gram chow (Plavix®, Sanofi-Aventis, Gouda, The Netherlands) in order to prevent platelet aggregation and to mimick the human clinical setting. Four weeks after stenting, rats were anesthesized and heparinized systemically with 500 IU i.v. (Leo Pharma, Breda, The Netherlands). The stented aortas were harvested, fixed in 4% formaldehyde and embedded in methylmetacrylate for further histological analysis. During stenting, 3 non-diabetic control BBDP rats died because of perforation of the aorta, resulting in an overall mortality of 17%. In addition, 2 stents from long-term diabetic BBDP rats were lost during histological processing. Overall, stents from 7 diabetic and 6 non-diabetic BBDP rats were included for histological analysis.

Quantification in-stent restenosis (ISR)

To quantify the severity of ISR, computerized morphometric analysis was performed on Lawson (elastin)-stained sections obtained from the proximal, middle, and distal parts of each stent. The neointimal area was calculated by measuring the total area within the internal elastic lamina and the remaining lumen using an Olympus BX-50F4 microscope equipped with an Olympus c-3030 zoom digital camera and Olympus DP-Soft version 3.0 software (Olympus, Tokyo, Japan). Total surface neointima (in μm^2) was then calculated by subtracting surface remaining lumen from the total surface area within the internal elastic lamina.

Quantification vessel injury

The mean vessel injury score was determined as described previously[29] using a method originally developed by Schwartz *et al.*[34] Vessel injury at every stent strut within a cross section (9-10 struts/cross section) was determined based on the anatomic vessel structures penetrated by each strut. This value ranged from 0 (least injury) to 3 (most injury). For each cross section the mean injury score was calculated. From each stent 6-8 cross sections (taken from the proximal, middle, and distal parts of the stent) were analyzed. The total mean injury score was expressed as the mean of the injury scores of all cross sections analyzed within one stent.

Statistical analysis

Data are expressed as mean ± SEM. Differences between two groups were analyzed for statistical significance using an independent samples *t* test. All p-values were two-tailed, and a p-value <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.00 for Windows (GraphPad Software Inc., USA).

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Results

Glucose homeostasis in long-term diabetic BBDP rats

Diabetes incidence in the BBDP colony maintained at the University Medical Center Groningen is 80-90%. Median age of diabetes onset in the BBDP rats (n=7) that were stented and included for histological analysis was 82 days (Table 1). Insulin treatment using insulin-releasing implants resulted in an almost permanent hyperglycaemic state (blood glucose >8 mmol/L) in all diabetic rats. In individual rats blood glucose oscillations were observed that varied between 8 and 27 mmol/L. Depending on the blood glucose levels (>20 mmol/L or 15-20 mmol/L in the presence of substantial weight loss [>10% weight loss compared with previous measurement]), rats received an insulin implant. Time between reimplantations varied between 5 and 35 days. As non-diabetic age-matched controls, 6 thymectomized BBDP rats were included. Figure 1 shows the blood glucose oscillations observed in an individual diabetic and non-diabetic BBDP rat during a time frame of 140 days during which the diabetic rat received 9 insulin implants. The median age at stenting was 270 and 298 days for the diabetic and non-diabetic BBDP rats, respectively (not significantly

Table 1. Characteristics of the diabetic (DM) and thymectomized non-diabetic (non-DM) BBDP rats that were stented and evaluated for the development of in-stent restenosis.

	N	gender ○ / ○	diabetes onset (median in days)	age at stenting (median in days)	diabetes duration at stenting (median in days)	serum creatinine [a] (mean ± SEM in µmol/L)
non-DM BBDP	6	4 / 2	N.A.[b]	298 [270 – 412][c]	N.A.[b]	51 ± 7
DM BBDP	7	3 / 4	82 [72 – 140][c]	270 [270 – 290][c]	198 [150 – 204][c]	56 ± 9

[a] serum creatinine levels were determined 2 weeks prior to stenting
[b] N.A.: not applicable
[c] values between brackets indicate minimum and maximum values

different), translating in a median diabetes duration of 198 days (Table 1). During the total follow-up period till stenting mean blood glucose level after diabetes onset in diabetic BBDP rats was 15.0±0.4 mmol/L compared with 5.3±0.1 mmol/L in thymectomized non-diabetic BBDP rats (Figure 2A, $p<0.001$). As increased HbA_{1c} levels have been associated with increased cardiovascular risk in human diabetics, we questioned whether long-term diabetes in our BBDP rat model was also associated with increased HbA_{1c} levels. To this end, HbA_{1c} levels were determined in a separate group of long-term diabetic BBDP rats (n=5) with a mean diabetes duration of 249±50 days which was not statistically different from the mean diabetes duration in the stented BBDP rats (217±7 days) at sacrifice. For comparison, HbA_{1c} levels were determined in spontaneously protected non-diabetic BBDP rats (n=2) and non-diabetic BBDR/Wor rats (n= 6). As shown in Figure 2B, long-term diabetes resulted in significantly increased HbA_{1c} levels (11.9±0.2 DCCT-HbA_{1c} %) compared with age-matched non-diabetic BB rats (5.1±0.1 DCCT-HbA_{1c} %).

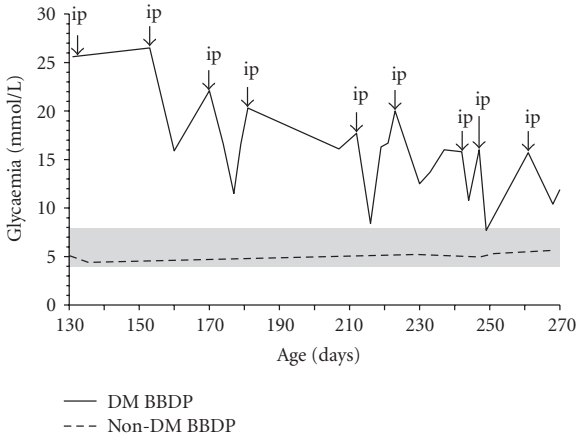

Figure 1. Oscillations in blood glucose levels in a representative diabetic (DM) BBDP rat treated with insulin pellets implanted subcutaneously. During a follow-up period of 140 days the DM BBDP rat received multiple insulin pellet implants (ip) guided by rapid development of hyperglycaemia (solid line) and drop in body weight (not shown). For comparison, glucose levels in an age-matched thymectomized non-DM BBDP rat are shown. The grey area indicates the normoglycaemic range (4-8 mmol/L).

Renal function in long-term diabetic BBDP rats

Two weeks before stenting part of the long-term diabetic and non-diabetic BBDP rats were housed in metabolic cages for 24 hrs to collect urine. Long-term diabetes and the associated permanent hyperglycaemic state resulted in a 6-fold increased ($p<0.001$) urinary volume compared with non-DM BBDP rats (Figure 3A). This was accompanied by severe polydipsia (not shown). Furthermore, total urinary protein excretion (Figure 3B, $p<0.01$) and urinary creatinine excretion (Figure 3C, $p<0.001$) were significantly increased in long-term DM BBDP rats compared with non-DM BBDP rats. Increased urinary creatinine excretion in DM BBDP rats suggests the presence of glomerular hyperfiltration which is commonly observed in diabetics. Hyperfiltration was supported by the slightly increased creatinine clearance rates observed in the long-term DM rats (1.7±0.3 ml/min in DM *vs.* 1.0±0.2 ml/min in non-DM, not significant). Despite the presence of proteinuria and hyperfiltration in long-term DM BBDP rats, plasma creatinine levels in these rats were similar to the levels detected in age-matched non-DM BBDP rats indicating preserved renal function in the presence of diabetes (Table 1). Histological analysis of PAS-stained renal sections did not reveal increased interstitial and glomerular matrix expansion in long-term DM BBDP rats (not shown).

Enhanced in-stent restenosis in long-term diabetic BBDP rats

In total, 18 rats (9 DM and 9 non-DM BBDP rats) received a bare metal stent (Figure 4A) in the abdominal aorta. During the stenting procedure, 3 non-DM BBDP rats died because of perforation of the aorta (mortality rate of 17%). Age of the DM and non-DM BBDP rats at stenting was similar between both groups (Table 1, median age respectively 270 and 298 days, not significantly different). Diabetes duration (median) in DM BBDP rats at stenting was 198 days (Table 1). Stents were harvested 28 days after stenting. Stents

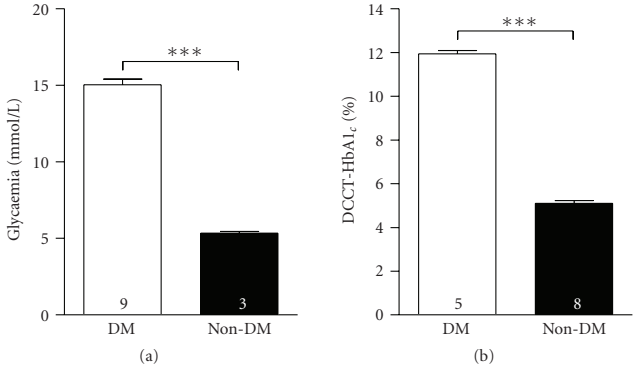

Figure 2. Blood glucose and HbA_{1c} levels are increased in long-term diabetic (DM) BBDP rats. (A) Average blood glucose level during the entire follow-up period starting at diabetes onset until sacrifice (ranging from 150 to >200 days) (white bar). For comparison, mean blood glucose levels in age-matched non-DM BBDP rats are shown (black bar). (B) Hyperglycaemia in long-term diabetic BBDP rats is associated with increased glycated haemoglobin (HbA_{1c}) levels (white bar) compared with non-DM rats (black bar). Data are expressed as the mean ± SEM (***$p<0.001$). Values within bars indicate the number of rats analyzed. DM = long-term diabetic BBDP rats, Non-DM = non-diabetic rats.

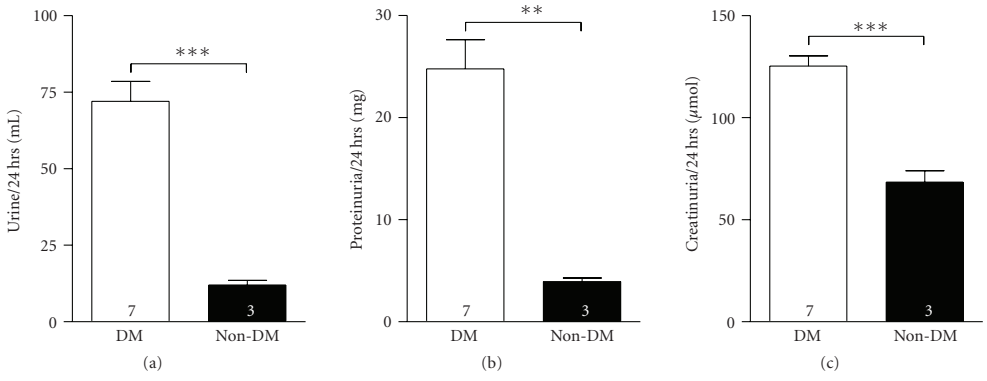

Figure 3. Long-term diabetes in BBDP rats is associated with significant polyuria (A), proteinuria (B) and creatinuria (C). Two weeks prior to stenting rats were housed in metabolic cages and 24 hr urine samples were collected and analyzed as described in *Materials and Methods*. Data are expressed as the mean ± SEM (**p<0.01, ***p<0.001). Values within bars indicate the number of rats analyzed. DM = long-term diabetic BBDP rats, Non-DM = non-diabetic rats.

from 2 DM BBDP rats were lost during histological processing. Finally, stents from 7 DM and 6 non-DM BBDP rats were histologically analyzed for the severity of ISR. At 28 days post-stenting in both non-DM and DM BBDP rats development of ISR was detected which was characterized by neointima formation surrounding the stent struts. Figure 4 D/E and Figure 4 F/G show representative photomicrographs of ISR in respectively non-DM and DM BBDP rats. Quantitative analysis revealed that long-term DM resulted in a 32% increase in surface neointima compared with non-DM BBDP rats (Figure 4B, p=0.02). This increase in neointima formation in DM BBDP rats was not associated with an increased mean injury score (Figure 4C, p=0.957).

Discussion

Diabetes mellitus (DM) is associated with increased risk for the development of in-stent restenosis (ISR).[15-17] Underlying pathogenetic mechanisms are as yet unknown and adequate treatment modalities are lacking. In order to increase our insights into the molecular and cellular mechanism(s) underlying Type 1 DM-enhanced development of ISR a clinically relevant rodent model might be of great value. Yet a model as such is not available. We therefore tested the hypothesis that long-term spontaneously diabetic hyperglycaemic BBDP rats develop enhanced ISR and may be used as a suitable model to study the molecular and cellular mechanism(s) involved in DM-enhanced development of ISR.

The hyperglycemic syndrome in BBDP rats develops spontaneously due to a disturbed balance in autoreactive and regulatory T cells.[26-28] The BBDP rat has been suggested to represent the best rodent model for human Type 1 DM as in this model diabetes manifests during adolescence and involves an autoimmune disorder without the need of exogenous intervention.

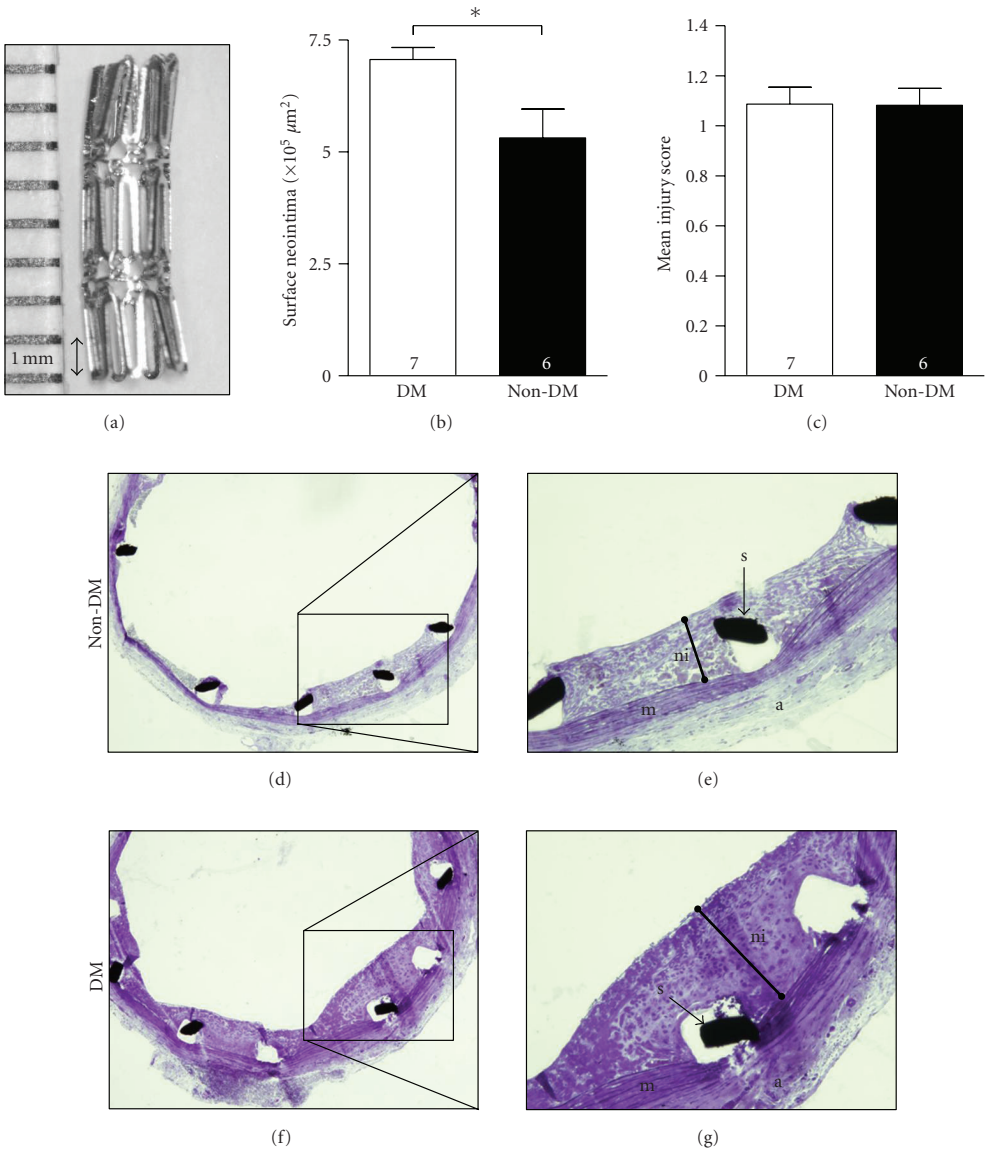

Figure 4. Long-term diabetes in BBDP rats is associated with enhanced in-stent restenosis 4 weeks after stenting in the abdominal aorta. (A) Photograph of a BeStent^{tm} 2 bare metal stent used for implantation. The inflation balloon has been removed. (B) Surface area neointima present 28 days after stenting in long-term DM (white bar) and non-DM (black bar) BBDP rats. (C) Mean injury score at 28 days after stenting in long-term DM (white bar) and non-DM (black bar) BBDP rats. Data are expressed as the mean ± SEM (*$p<0.05$). Values within bars indicate the number of rats analyzed. DM = long-term diabetic BBDP rats, Non-DM = non-diabetic rats. (D, E) Representative photomicrographs (Lawson staining) of stented abdominal aorta's from a thymectomized non-diabetic (non-DM) BBDP rat 28 days after stenting. (F, G) Representative photomicrographs (Lawson staining) of stented abdominal aorta's from a diabetic (DM) BBDP rat 28 days after stenting. E and G (magnification x100) are higher-power magnifications of the framed area's shown, in respectively, D and F (magnification x40). Abbreviations: a: adventitia; m: media; ni: neointima; s: stent strut.

To achieve suboptimally-controlled DM with manifest hyperglycaemia and elevated HbA_{1c} levels, recent onset diabetic BBDP rats were treated with insulin using slow release insulin implants. By doing so, we were able to maintain DM BBDP rats in a rather permanent hyperglycaemic state for at least 8 months. The hyperglycaemic state was associated with increased HbA_{1c} levels as well as polyuria, polydipsia, proteinuria and glomerular hyperfiltration. Severity of proteinuria was within the range reported by Cohen *et al.* who also determined protein excretion in long-term DM BBDP rats that were treated with daily insulin injections.[35] As older age is associated with increased restenosis rates independent of DM[17], for our study it was important to include age-matched non-diabetic controls rather than pre-diabetic young BBDP rats. In order to prevent diabetes development in BBDP rats thymectomy was performed at the age of 21 days as we described previously.[30] DM and non-DM BBDP rats were followed for 7 months after which they received a bare metal stent implanted in the abdominal aorta. Our results clearly demonstrated that long-term DM significantly enhanced the development of ISR by 32% compared with non-DM age-matched thymectomized BBDP rats. This enhanced development of ISR in diabetic BBDP rats was not associated with increased vascular injury *i.e.* increased penetration of the stent struts through the internal elastic lamina into the medial layer. The severity of ISR was previously shown to be positively correlated with the mean injury score.[29] However, in the same study we demonstrated differences in severity of ISR between bare metal and sirolimus-eluting stents, which was independent of the mean injury score. Our data suggest that T1DM enhances the development of ISR by factors other than direct vascular injury.

The current study was performed as a proof of concept to demonstrate that long-term T1DM in BBDP rats indeed enhances the development of ISR following stenting in the abdominal aorta. Although we did not study the underlying mechanism(s) of enhanced ISR in diabetic BBDP rats yet, we suggest that long-term diabetes increases the proliferative and migratory capacity of medial and neointimal smooth muscle cells, thereby facilitating neointima formation.[36,37] In addition, reduced endothelial repair capacity in diabetic BBDP rats might have contributed to enhanced ISR[38], but this needs to be determined.

The major advantage of the BBDP model to study the development of ISR over other T1DM rodent models is the fact that diabetes develops due to autoimmune-mediated destruction of pancreatic islets without having the toxic side effects of the use of streptozotocin or alloxan. By using slow release insulin implants we showed that long-term studies are feasible without the need of daily insulin injections. A possible weakness of the model is that BBDP rats are T cell lymphopenic and are, in that respect, not fully immunocompetent. However, despite T cell lymphopenia BBDP rats do develop autoimmune diabetes which is mediated by autoreactive T cells indicating that functional T cells are present in BBDP rats. Whether T cells are pivotal in the development of ISR is however unclear. Percutaneous transluminal coronary angioplasty has been shown to induce T cell activation in a small cohort of 10 patients with stable angina. Patients that developed restenosis had higher T cell activation levels than patients that did not develop restenosis.[39] However, treatment with the calcineurin inhibitor cyclosporine to prevent

T cell activation did not reduce the development of restenosis in rabbits.[40] We therefore assume that the presence of T cell lymphopenia in BBDP rats has not been of major influence on the development of ISR.

Conclusions

The BBDP rat model for Type 1 diabetes is suitable for studies on the long-term effects of hyperglycaemia on the development of ISR. Long-term diabetes significantly increased the development of ISR. To our opinion, future studies aiming at the identification of the molecular and cellular mechanisms involved as well as on testing the efficacy of novel therapeutic interventions in this model are warranted.

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8

Angiogenic sprouting from the aortic vascular wall is impaired in the BB rat model of autoimmune diabetes

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Abstract

Background. Diabetes is associated with impaired neovascularization leading to reduced revascularization of ischemic tissue and impaired wound healing. Neovascularization is a complex process, involving resident endothelial cells as well as circulating endothelial progenitor cells. Endothelial progenitor cells in diabetes were previously shown to be numerically reduced and functionally impaired. We hypothesize that diabetes also has a long-term effect on angiogenic cells residing in the vessel wall. To test this hypothesis, angiogenic sprout formation from *ex vivo* cultured aortic rings isolated from diabetic and non-diabetic BioBreeding (BB) rats was assessed.

Methods. Diabetes prone BB (BBDP) rats spontaneously develop autoimmune diabetes and were suboptimally treated with insulin by subcutaneous implantation of slow-release insulin-pellets. Neonatally thymectomized BBDP rats, pre-diabetic BBDP rats and diabetes resistant BBDR rats served as non-diabetic controls. After follow-up thoracic aortas were harvested and cultured *in vitro* in Matrigel to induce sprout formation. Sprout length was quantified after 4, 7, 10 and 14 days of culture. The total number of sprout-derived cells was measured and *in vitro* proliferative capacity of sprout cells was quantified. Finally, expression of Flk-1, CD31 and smooth muscle α-actin on sprout cells was determined.

Results. Mean blood glucose levels in diabetics was significantly elevated compared with non-diabetics. Both long-term and short-term diabetes significantly reduced sprout formation ($p<0.05$ *vs.* non-diabetics). Reduced sprout length in diabetics was reflected by significantly reduced numbers of sprout cells that could be isolated ($p<0.05$ *vs.* non-diabetics). Isolated sprout cells from diabetics revealed significantly reduced proliferative capacity after *in vitro* culture ($p<0.05$ *vs.* non-diabetics). Immunofluorescent staining indicated an endothelial phenotype of both freshly isolated and *in vitro* cultured sprout cells as indicated by CD31 and Flk-1 expression and absence of smooth muscle α-actin expression.

Conclusions. Diabetes in BB rats impairs angiogenic sprouting from cells residing in the vascular wall, independent of effects on circulating cells or circulating angiogenic/anti-angiogenic factors. The angiogenic impairment of diabetic sprout cells is, to some extent, imprinted upon the cells.

Introduction

Diabetes mellitus is associated with impaired neovascularization leading to reduced revascularization of ischemic tissue, impaired wound healing, embryonic vasculopathy, and organ transplant rejection in diabetic patients.[1,2] On the other hand, pathologically enhanced neovascularisation is also observed in diabetes, contributing to diabetic retinopathy, diabetic nephropathy, and possibly atherosclerotic plaque destabilization.[1,2] Neovascularization is a complex process, involving growth factors, cytokines, and both resident endothelial cells as well as circulating cells. Circulating factors in the diabetic milieu directly influence neovascularization. Hyperglycemia decreases endothelial cell proliferation *in vitro*[3] and myocardial interstitial fluid from dogs with experimental diabetes impairs angiogenic tube formation by cultured endothelial cells.[4] The diabetic milieu also influences circulating cells. Monocytes from diabetic patients respond poorly to angiogenic chemotactic factor VEGF-A[5] and circulating angiogenic endothelial progenitor cells were previously shown to be numerically reduced and functionally impaired in diabetes.[6]

It is currently unclear what the effect of diabetes is on angiogenic cells residing in the vessel wall, which are potent contributors to neovascularization.[7] We hypothesized that diabetes would attenuate their angiogenic capacity and therefore assessed angiogenic sprout formation from *ex vivo* cultured aorta rings isolated from diabetic and non-diabetic diabetes-prone BioBreeding (BBDP) rats. We furthermore determined the phenotype of the outgrowing sprout cells. This sprouting-assay provides a tool to specifically study the long-term effect of diabetes on the angiogenic capacity of cells residing in the vessel wall, thereby excluding the direct influence of both circulating factors and circulating cells.

Material and Methods

Diabetes Prone BioBreeding BBDP rats

Both male and female BBDP/Wor rats were used. BBDP rats spontaneously develop autoimmune diabetes due to the absence of regulatory T cells and are used to model human type 1 diabetes.[8] Age-matched neonatally thymectomized BBDP rats and pre-diabetic BBDP rats served as non-diabetic controls. All animal use was in accordance with the guidelines of the "Principles of laboratory animal care" and the Animal Ethics Committee of the University Medical Center Groningen. In this study, two experimental groups were included. Group 1 (long-term group) had a long diabetic course (~45 wks diabetes) and consisted of 15 diabetic BBDP rats and 8 age-matched neonatally thymectomized non-diabetic BBDP rats. Group 2 (short-term group) had a relatively short diabetes duration and consisted of 8 diabetic BBDP rats (~6 wks diabetes; ~17 wks of age) and 7 pre-diabetic BBDP rats (8 wks of age). Diabetic rats were suboptimally treated with insulin by subcutaneous implantation of slow-release Linplant insulin-pellets (LinShin, Scarborough, Canada).

Aortic ring sprouting assay

Aortas were harvested from the diabetic and non-diabetic BBDP rats under sterile conditions and flushed with saline to remove residual blood. Then ~1mm (group 1) and 0.65mm (group 2) thick aortic rings were transversally cut manually or using an automatic tissue chopper (McIlwain Tissue Chopper, The Mickle Laboratory Engineering Co. LDT., Gomshall, Surrey, England), respectively. Aortic rings were centrally positioned in the wells of 96-well tissue culture plates containing cold liquefied BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Alphen aan den Rijn, The Netherlands), and overlaid with EC medium (RPMI1640 medium containing 2 mM L-glutamine, 50 µg/ml endothelial cell growth factor (ECGF), 5 units/ml heparin, 100 U/ml penicillin, 100 µg/ml streptomycin and 20% fetal calf serum). Cultures were performed in duplicate (group 1) or triplicate (group 2). Plates were then placed at 37°C 5% CO_2, allowing the matrix solution to solidify. Sprout length in the long-term diabetic group (group 1) was measured after 10 days of culture. In the short-term diabetic group (group 2), sprout length was measured after 4, 7, 10 and 14 days of culture in order to evaluate kinetics of sprout formation, with having medium replenished at the moment of measurements. Sprout length was quantified (expressed in arbitrary units) as mean maximal sprout length from the perimeter of the aortic ring to the most distal tip of the angiogenic sprout in four quadrants of each aortic ring under an inverted microscope at 40-fold magnification using a microscopic grid.

Sprout cell isolation

To evaluate the absolute number of sprout cells, sprouts growing in Matrigel™ from the short-term diabetics and pre-diabetics (group 2) were isolated and counted. Medium and aorta rings were removed from the Matrigel™ cultures after which remaining sprouts and Matrigel™ were incubated with dispase (BD, Alphen aan den Rijn, The Netherlands, 2.5 caseinolytic units/well) at 37°C, 5% CO_2 for 2 hours to ensure complete dissociation. Cultures were performed in triplicate and after enzymatic digestion the total solution obtained from triplicate cultures was diluted in 10 ml PBS. Absolute numbers and viability of isolated sprout cells were determined using a haemocytometer and Trypan Blue exclusion. Viability was consistently >98% (not shown). Cell counting was performed after 4, 7, 10 and 14 days of culture in Matrigel™. Cells isolated after 10 days of culture in Matrigel™ were either replated in fresh EC medium for continued cell culture (Evaluation of sprout cell proliferative capacity: described below) or resuspended in PBS + 2.5% BSA for cytospots. Cytospots were prepared by spinning a total of $5x10^4$ isolated cells onto glass-slides (5 min., 550 rpm) using a Shandon Cytospin 4 (Thermo Fisher Scientific).

Evaluation of sprout cell proliferative capacity

To determine the proliferative status of sprout cells, cytospots ($5x10^4$ cells/spot) of cells isolated after 10 days of culture in Matrigel™ were immunostained for Ki67, a marker which is expressed during all active phases of cell replication but not in resting cells. Cytospots were acetone-fixed for 12 min and dried for 1 hr at room temperature (RT). Blockade of endogenous peroxidase (0.03% H_2O_2 in PBS for 10 min) was followed by

incubation (1 hr, RT) with α-Ki67 monoclonal antibody (DakoCytomation Denmark A/S, Glostrup, Denmark). Subsequently, cytospots were incubated (30 min, RT) with horseradish peroxidase-conjugated rabbit-α-mouse antibody (DakoCytomation Denmark A/S, Glostrup, Denmark) followed by visualisation with diaminobenzidine (DAB, Sigma-Aldrich, Zwijndrecht, The Netherlands). Cells were counterstained with hematoxylin and coverslipped in Depex mounting medium. All $Ki67^+$ cells per cytospot ($5x10^4$ cells) were counted.

To further evaluate the proliferative capacity of isolated sprout cells after replating, $2x10^4$ cells/well were seeded in EC medium in 6-well tissue culture plates that were precoated (1 hr) with 1 ml/well Matrigel™ (1 mg/ml). Cells were cultured for 96 hrs (4 days) after which they were trypsinized and counted using a haemocytometer and Trypan Blue exclusion. In some experiments cells were cultured in Matrigel™-coated 8-wells chamberslides (Lab-Tek™, Nunc, VWR International B.V., Amsterdam, The Netherlands) for phenotypic analysis.

Phenotypic analysis of sprout cells

To demonstrate an EC-phenotype of isolated sprout cells, cytospots were stained using the following antibodies: α-CD31 (clone TLD-3A12, mIgG1, BD Pharmingen), α-Flk-1 (clone A-3, mIgG1, Santa Cruz Biotechnology), mAb HIS43[9] (own produced hybridoma tissue culture supernatant, mIgG1) and α-alpha-SMA (vascular smooth muscle cells clone 1A4, mIgG2a, DakoCytomation A/S). After aceton fixation (12 min, -20°C) cytospots were incubated with primary monoclonal antibodies (1 hr, RT). Cytospots were then incubated (30 min, RT) with TRITC-conjugated goat-α-mouse IgG1 and FITC-conjugated goat-α-mouse IgG2a antibodies (both from Southern Biotechnology Associates, Birmingham, Alabama, USA) diluted in PBS + 3% normal rat serum. After nuclear staining with DAPI cytospots were embedded in Citifluor. Cytospots were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems Nederland B.V., Rijswijk, The Netherlands).

Statistical analysis

Data are expressed as mean±SEM and were analyzed using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, USA) using a two-tailed Students' t-test. A p-value of <0.05 was considered statistically significant.

Results

Diabetes onset and glycaemic control

Non-thymectomized BBDP rats developed diabetes with a median onset of diabetes at the age 84 days (group 1; long-term) and 75 days (group 2; short-term), respectively. Age-matched control thymectomized BBDP rats did not become diabetic. In group 1 mean follow-up time was 324 and 386 days for the diabetic and thymectomized non-diabetic BBDP rats, respectively. Mean blood glucose level after long-term diabetes was 15.1±0.3 mmol/L versus 5.3±0.1 mmol/L in thymectomized non-diabetic controls (Figure 1A, $p<0.0001$). In group 2 mean follow-up time was 120 and 56 days for the short-term diabetic

and pre-diabetic BBDP rats, respectively. Mean blood glucose level after diabetes onset was 20.8±1.0 mmol/L versus 7.4±0.3 mmol/L in pre-diabetic controls.

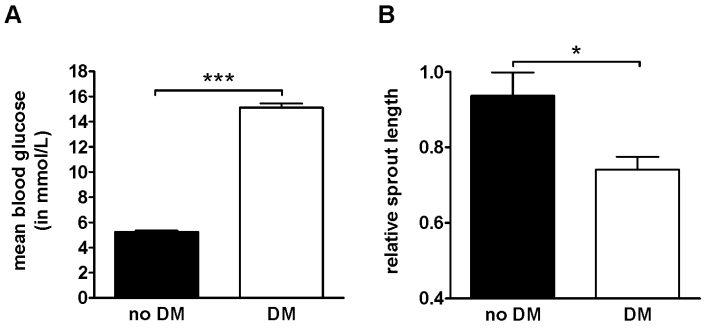

Figure 1. Long-term diabetes (~45 wks) impairs aortic sprouting. (**A**) Mean blood glucose levels in group 1: long-term diabetics (DM, n=15) and age-matched thymectomized non-diabetic controls (no DM, n=8). (***p<0.0001) (**B**) Quantification of the relative sprout length from aortic rings revealed significantly reduced (*p<0.05) sprout formation *in vitro* after exposure to hyperglycemic conditions in diabetic BBDP rats *in vivo* compared to age-matched thymectomized non-diabetic control BBDP rats.

Angiogenic sprouting from aorta rings is impaired in diabetic animals

In both diabetic and non-diabetic BBDP rats we observed sprouts starting to emerge from the vascular wall and growing outward after 4 days of culture in Matrigel™. All rings generated sprouts of comparable morphology although sprout length and sprout density was reduced in the diabetic rats. In group 1 (long-term group) maximal sprout length in aortic rings measured at day 10 was significantly lower in diabetic rats than those from thymectomized non-diabetic controls (0.94±0.06 vs. 0.77±0.03, p<0.05, Figure 1B). In order to determine the kinetics of sprout development, sprout length was determined in the short-term diabetics and pre-diabetic BBDP rats at 4, 7, 10 and 14 days after culture in Matrigel™. Figure 2A shows representative photomicrographs depicting reduced sprout formation in rings from diabetic rats (right panel) compared with rings from pre-diabetic rats (left panel). Quantitative analysis revealed reduced sprout length in diabetic rats at 4, 7 and 10 days after culture in Matrigel™ reaching the level of statistical significance at day 4 (** p<0.01) and day 10 (* p<0.05) compared with pre-diabetic rats (Figure 2B). After 14 days of culture in Matrigel™ sprouts had reached the border of the well, and thereby maximal sprout length, in rings from both diabetic and pre-diabetic rats.

Our microscopic analysis of developing sprouts suggested reduced sprout density in rings obtained from diabetic rats. Reduced sprout length and sprout density is anticipated to result in the presence of decreased numbers of sprout cells in diabetic rats compared with pre-diabetic rats. To test this assumption we determined the absolute number of cells that could be isolated from sprouts that had grown out of aortic rings from short-term diabetic and pre-diabetic BBDP rats. Cells were isolated by enzymatic digestion using dispase followed by counting using a haemacytometer. As shown in Figure 2C absolute numbers of cells isolated from sprouts from diabetic rats were significantly reduced (at days

4, 7 and 14) compared with the numbers of cells isolated from sprouts from pre-diabetic rats. (* p<0.05, ** p<0.01, *** p<0.0001)

Aortic sprout cells from diabetic rats have a lower proliferative status

A possible explanation for reduced sprout length and density in diabetic rats is a reduced proliferative capacity of sprout cells. To test this possibility we determined the proliferative status of sprout cells by performing an immunostaining for the proliferation marker Ki67 on cytospots of cells isolated from sprouts that were cultured for 10 days in Matrigel™. Figure 3A shows the nuclear staining for Ki67 in dividing cells. Compared with pre-diabetic controls, diabetic rats had significantly (** p<0.01) less Ki67-positive cells per $5x10^4$ spotted cells (Figure 3B) suggesting a lower proliferation status of sprout cells derived from diabetic rats.

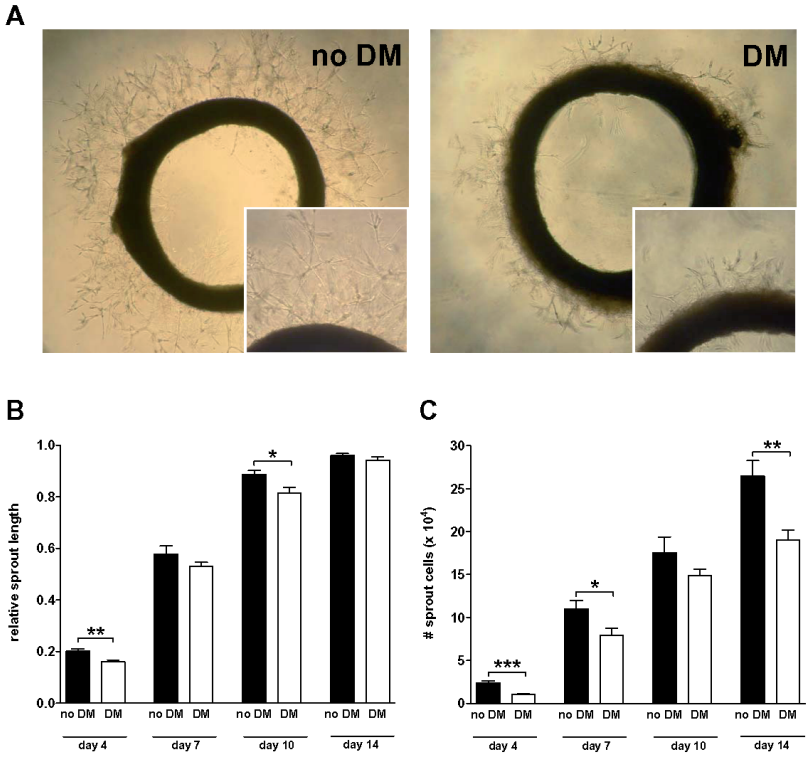

Figure 2. Kinetics of *in vitro* sprout-formation in aortic rings from short-term (~6 wks) diabetic (DM) and pre-diabetic (no DM) BBDP rats. (A) Representative photomicrographs of aorta rings from pre-diabetic (no DM; left panel) and diabetic (DM; right panel) BBDP rats after 4 days of culture in Matrigel™. (B) Quantification of sprout length from aortic rings revealed reduced sprout formation in vitro after exposure to hyperglycemic conditions in diabetic BBDP rats in vivo (compared to pre-diabetic BBDP rats) after 4, 7, and 10 days of culture in Matrigel™. (* p<0.05, ** p<0.01) (C) Quantification of the absolute numbers of sprout cells revealed significantly reduced numbers in diabetic rats (DM) compared with pre-diabetic (no DM) control rats after 4, 7 and 14 days of culture. (* p<0.05,** p<0.01, *** p<0.0001)

Aortic sprout cells from diabetic rats maintain their low proliferation status in vitro

To study whether sprout cells derived from diabetic rats maintain their reduced proliferative capacity under normoglycemic conditions *in vitro*, a fixed number of $2x10^4$ cells was seeded and cultured in EC medium in 6-well plates. After 4 days of culture seeded cells had differentiated into elongated cells that expressed Flk-1 (VEGFR2) suggesting an endothelial phenotype. Occasionally SMA^+ myofibroblasts were detected in these cultures (Figure 3C). After 4 days of culture, cells were trypsinized and counted. As shown in Figure 3D, *in vitro* culture of sprout cells obtained from diabetic rats resulted in significantly ($p<0.05$) reduced cell numbers compared with pre-diabetic controls. These data indicate that sprout cells isolated from aortic rings obtained from diabetic rats have reduced proliferative capacity which persists under normoglycemic conditions *in vitro*.

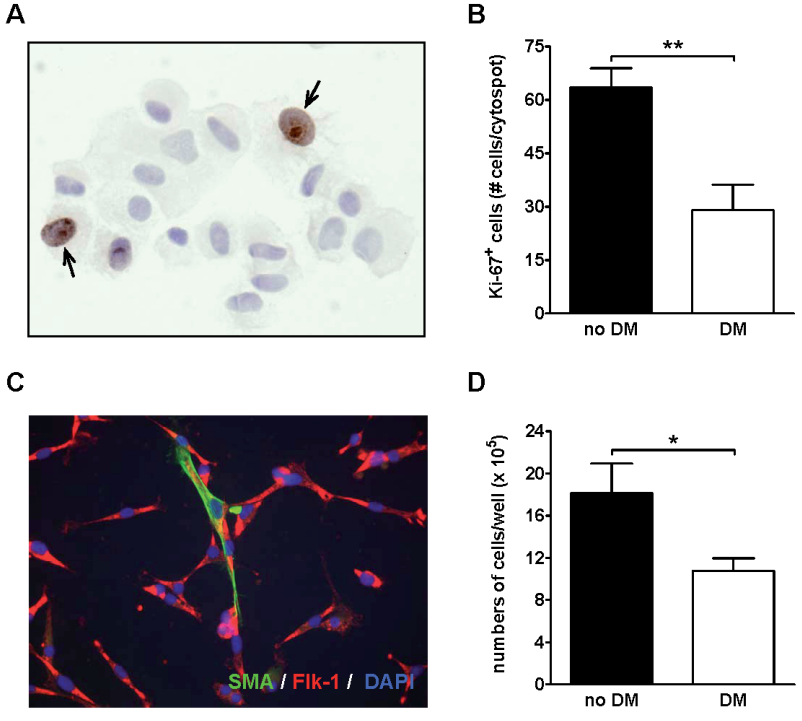

Figure 3. Sprout cells from short-term diabetic BBDP rats have a decreased proliferation status. (A) Representative photomicrograph of cytospots of sprout cells isolated after 10 days of culture in Matrigel™ and stained with the proliferation marker Ki67. Arrows indicate proliferating Ki67+ cells. (B) Quantification of Ki67+ cells on cytospots ($5x10^4$ cells/spot) shows significantly reduced numbers of positive cells in diabetic rats (DM, n=7) compared with pre-diabetic rats (no DM, n=7). (** $p<0.01$) (C) Virtually all *in vitro* expanded isolated sprout cells express Flk-1 (VEGFR2) (red) whereas occasionally smooth muscle α-actin (α-SMA)-positive myofibroblasts were detected (green) (magnification x400). (D) *In vitro* culture of isolated sprout cells for 4 days in EC medium shows significantly reduced expansion of cells derived from short-term diabetic rats (DM, n=8) compared with pre-diabetic control rats (no DM, n=7).

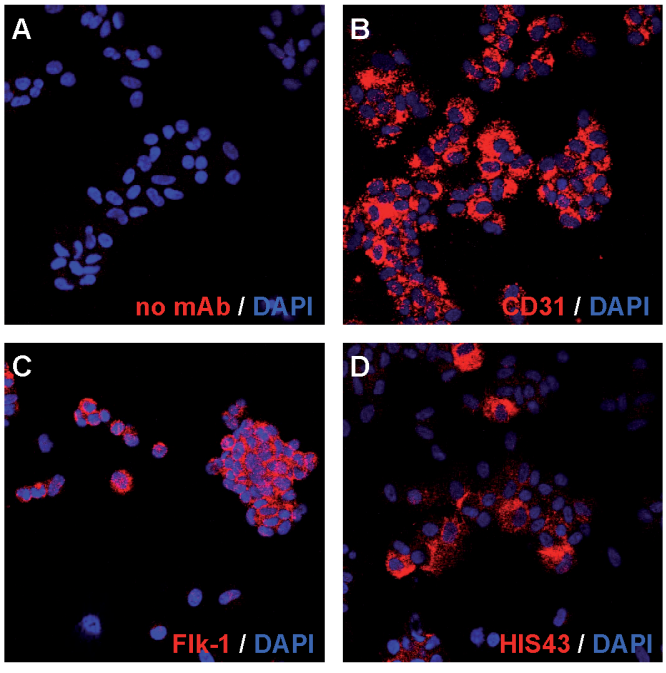

Figure 4. Immunofluorescent staining of sprout cell cytospots (cells isolated after 10 days of culture in Matrigel™) indicates an endothelial cell phenotype. (A) negative control (no primary antibody added), (B) CD31 (PECAM) expression, (C) Flk-1 (VEGFR2) expression, (D) protein expression recognized by HIS43. (magnification x630).

Aortic sprout cells express endothelial markers

To demonstrate that the sprout cells are predominantly endothelial cells, additional immunofluorescent stainings were performed on cytospots of sprout cells isolated after 10 days of culture on Matrigel™ using antibodies against the EC-markers CD31, Flk-1 (VEGFR2) and the antigen recognized by mAb HIS43. As shown in Figure 4, most sprout cells stained positive for CD31 and Flk-1 and to a somewhat lesser extent HIS43. Occasionally a SMA^+ myofibroblast was detected. Similar results were obtained on isolated sprout cells that were cultured for another 4 days (not shown). Together, these data indicate that the vast majority of the sprout cells are endothelial cells.

Discussion

Understanding how diabetes affects neovascularisation is important for the development of pro- or anti-angiogenic therapeutic strategies. Our data from isolated vessels show that the effects of diabetes on neovascularisation include impaired angiogenic sprouting from cells residing in the vascular wall, independent of effects on circulating cells or circulating angiogenic/anti-angiogenic factors. As the vascular rings and isolated sprout cells were cultured *ex vivo* under normoglycemic conditions, the angiogenic impairment of diabetic

sprouting cells is at least to some extent imprinted upon the cells. The model of aortic ring sprouting provides a method to specifically evaluate the effect of potential pro- or anti-angiogenic interventions at the level of the resident cells in the vascular wall. To what extent the impairment observed in this study *ex vivo* is physiologically relevant *in viv*o remains to be established.

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9

General discussion

Introduction

The aim of the studies described in this thesis was to gain more insight into the (cellular and molecular) mechanisms underlying the development of transplant arteriosclerosis (TA) and in-stent restenosis (ISR). TA and ISR represent two major macrovascular occlusive diseases for which no effective preventive and treatment strategies currently exist. Both TA and ISR are processes of inward vascular remodeling that develop long-term after allogeneic solid organ transplantation (particularly in heart and kidney) and after endovascular stenting, respectively. The vascular occlusions (*i.e.* neointima [NI]) in TA and ISR primarily consist of α-smooth muscle actin-expressing myofibroblasts or vascular smooth muscle cells (VSMCs). Both TA and ISR presumably result from vascular wall damage although the inciting damage-inducing events clearly differ. TA is the result of alloantigen- and non-alloantigen-mediated damage to graft vasculature whereas ISR results from mechanically-induced vascular damage. The foremost target for initial damage resulting in TA and ISR is most likely the endothelium, which plays an important role in the onset and progression of these diseases.[1-3] Insults to the endothelium lining the luminal surface of arteries prime the successive cascade of inflammatory, migratory and proliferative events culminating in obstructive NI formation. Endothelial cell (EC) loss must be associated with an efficient mechanism of concomitant regeneration in order to reestablish vessel integrity. This can be achieved by resident vascular EC proliferation (when sufficient reservoirs are available) and by recruitment of circulating and/or vascular wall-resident endothelial progenitor cells (EPCs). Although it is well established that proliferation and migration of VSMCs is a crucial event in intimal expansion, the anatomical origin of neointimal cells is still a matter of debate. In addition to medial (donor) origin of neointimal cells (as proposed in the "response to injury" hypothesis), also recruitment of recipient-derived cells ([recirculating] bone marrow [BM]-derived or vascular wall residing precursor cells) might contribute to NI formation. These recipient-derived cells are considered to originate from a population of Smooth Muscle Progenitor Cells (SMPCs). Diverse sources for neointimal VSMCs been described[4-6] including donor medial VSMCs, fibroblast-myofibroblast transdifferentiation (donor or recipient origin), adjacent recipient medial VSMCs (recipient origin), BM-derived SMPCs (recipient origin), and non-BM-derived SMPCs residing in the vascular wall (donor or recipient origin). Regardless of their exact origin, chemotactic factors are required to recruit the neointimal VSM ancestral cells towards the developing neointima. A range of chemokine pathways including SDF-1-CXCR4, fractalkine-CX3CR1, and MIPα/RANTES/MCP3-CCR1 are known to be involved in mobilization of different subtypes of pro-angiogenic cells, with direct influence on NI formation. Despite the advances made in understanding the cellular and molecular mechanisms involved in NI formation, the biology of progenitor cell recruitment needs to be further explored. Focus of this thesis was therefore to obtain more insight on post-injury endothelium regeneration, neointimal cell origin, their recruitment and proliferation, as well as the therapeutic potential of a peroxisome proliferator-activated receptor γ agonist in inhibiting NI formation. The results of the studies performed are described in detail in the previous chapters. Below, the data obtained will be discussed and put in a broader context.

Chapter 1 provided an overview of various aspects of vascular remodeling processes involved in macrovascular complications following solid organ transplantation (TA) and stenting (ISR). Similarities and differences in the pathogenetic mechanisms of TA and ISR development are presented. Special attention is paid to the central role of endothelium in the initiating phase of TA and ISR development, VSMC proliferation and the potential role of vascular progenitor cells in NI formation. **Chapter 2** shortly introduced the experimental work described in the subsequent chapters.

Part I: Transplant Arteriosclerosis (TA)

Susceptibility for TA correlates with neointimal VSMC proliferation and fibrocyte frequency

Development of TA is primarily the result of immune-mediated vascular damage in transplanted organs. TA is considered as the general histologic hallmark of chronic rejection.[7] Despite major progress achieved in preventing and treating acute rejection episodes relatively early after transplantation (<1 year post transplantation), long-term outcome in solid organ transplantation remains unsatisfactory.[8,9] Among transplant recipients transplanted with similar HLA-incompatible grafts, receiving similar immunosuppression and exposed to the same known risk factors, variation exists in both the rejection rate and long-term outcome. This variation has not been fully explained yet, but data indicate that both immune and non-immune-related factors might contribute in determining the different transplant outcomes in different individuals.[10-21] In addition to differences in preservation, ischemia-reperfusion, cytokine status of the recipient (due to *e.g.* infections, surgical trauma, use of blood-related products), clinical data suggest that different recipients might display different immune responses against an allograft (*i.e.* a different immunological responder status). Both a donor and a recipient origin of neointimal cells have been described. In the rat aorta transplant model, we and others showed that the cells forming the neointima are recipient-derived.[22-25] Based on this observation in **Chapter 3** the hypothesis that (genetically-determined) differences in neointimal VSMC proliferative capacity and recipient progenitor cell frequency are correlated with TA susceptibility was tested. To this end, host MHC and non-MHC-encoded determinants, intrinsic neointimal VSMC proliferative capacity, and the VSMC progenitor cell availability were analyzed in different donor-recipient combinations using the aortic transplant model in rats. Using Lewis (Lew) and Brown Norway (BN) rat strains as donors and recipients, the development of TA in time was analyzed. In contrast to Lew hosts, which gradually developed TA with time, BN hosts developed maximal TA as early as 4 weeks after transplantation compared with 24 weeks in Lew hosts and this difference was dependent on the presence of BN non-MHC encoded determinants. The severity of TA in both strains was similar eventually, indicating that the rate of TA development rather than the severity per se was strain dependent. The high TA responder status of BN recipients was clearly correlated with the intrinsic proliferative capacity of neointimal VSMCs. Neointimal VSMCs isolated from Lew allografts transplanted in BN recipients (VSMCs of BN origin) displayed markedly increased proliferative responses *in vitro* compared with neointimal

VSMCs isolated from BN allografts transplanted in Lew recipients (VSMCs of Lew origin). This increased proliferation rate of BN-derived neointimal VSMCs was associated with increased endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN) mRNA expression levels. This observation is in line with the reported temporal and spatial ESDN expression pattern described in vascular cell proliferation during vascular remodeling.[26] A few years ago, ESDN was reported as a marker for neointimal VSMC proliferation, being minimally expressed in normal arteries and significantly upregulated following alloimune or mechanical injury.[26] ESDN overexpression *in vitro* leads to a decrease in growth VSMC rate.[26] Conversely, knocking down ESDN in VSMCs leads to an increase in number of platelet-derived growth factor (PDGF, a potent VSMC mitogen) binding sites (PDGFRβ), favoring PDGF-induced VSMC proliferation.[27] ESDN affects the number of PDGF receptors on the VSMC surface by modulating PDGFRβ ubiquitination. As such, PDGFRβ ubiquitination, as a negative regulator of PDGFRβ signaling, is reduced by ESDN down-regulation, resulting in increased PDGFRβ signaling.[27] Therefore, ESDN might play a role as a negative regulator of growth-inducing signals and its upregulation in high TA-responders can be considered in this respect as an attempt to control proliferation by reducing the response to growth factors in a negative feedback loop. ESDN upregulation may be a downstream effect in the signaling events that lead to cell proliferation or, alternatively, may be directly or indirectly induced by cell proliferation.[26] Further studies are necessary to establish the role of ESDN on neointima formation *in vivo* and evaluate its therapeutic potential controlling pathological states where excessive cell proliferation plays a central role. Furthermore, the high TA responder status of BN recipients was correlated with the frequency of circulating fibrocytes which are considered as putative neointimal VSMC ancestral cells also known as SMPCs. Although SMPCs have been described in various vascular (circulating SMPCs, vascular wall resident SMPCs) and extravascular sites (bone marrow, adipose tissue), it is likely that there is a continuous movement between compartments, and many resident and extravascular non-BM derived SPMCs were once derived from the BM during ontogeny. Efforts are being made for further characterization of specific SMPC antigens to allow their identification. Various progenitor and stem-derived surface and cytoplasmic SMPC antigens like CD14, CD34, CD105, flt1, c-kit, sca-1 were described.[5] Several chemokine pathways promoting mobilization of neointimal cells are already known. Intimal, but not medial, VSMCs were shown to express higher levels of functional CC chemokine receptor-1 (CCR1), essential for neointimal recruitment, in human and murine TA.[28] CX3CR1 mononuclear cell population was shown to provide a source of SMPCs and the CX3CR1-fractalkine interaction *in vivo* is essential for SMPC differentiation of BM-derived progenitor cells at the vessel wall level.[29] Stromal cell-derived factor 1α (SDF-1α)/CXCR4 is another SMPC-mediated vascular repair axis, shown to influence cell migration towards injured organs.[30]

In our model, the alloreactive immune response causes massive apoptosis of the medial VSMCs, and promotes recipient-derived SMPC recruitment to the place of vascular injury, leading to NI formation. The increased fibrocyte frequency in BN rats was detected in both naïve and transplanted rats indicating that these differences are endogenously present.

The observation that BN rats are high TA responders compared with LEW rats is in line with previous findings obtained in a balloon injury model showing that BN rats develop more severe restenosis compared with Lew rats.[31] These data indicate that the high TA responder status of BN rats may reflect a general susceptibility of BN rats to develop more severe occlusive vascular disease. These results thus indicate that in the process of NI formation following transplantation both increased progenitor cell availability, and possibly also their recruitment to the site of vascular damage, as well as increased neointimal VSMC proliferation are positively correlated with the rate of TA development. Before transplantation, screenings of transplant recipients for high frequency of SMPC circulating levels could therefore potentially reveal the subjects with increased risk for neointima development in grafted organs.

Endothelial injury is considered the initiating event in TA development[1] and the presence of an intact endothelial monolayer, either by preserving the donor endothelial cells or by reendothelialization with host-derived cells, supposedly leads to reduced TA development and prolonged graft survival. Therefore, we analyzed host-mediated endothelial repair, *i.e.* host EC-chimerism in the early post-transplant period. In line with the studies showing that increased levels of host EC-chimerism were associated with more severe vascular damage in renal allografts[32,33], we showed that the high BN TA responder-status was associated with enhanced host EC-chimerism when compared with the low Lew TA responder-status. These data indicate that enhanced EC-chimerism is associated with worse outcome, which might reflect an earlier and more aggressive acute vascular rejection and therefore a more severe vascular damage. In addition, reduced necessity for an accelerated reendothelialization in BN grafts transplanted in Lew recipients can reflect a lower vulnerability of BN endothelium to transplantation-related events like ischemia/reperfusion injury. Thus, therapies aiming at preservation of graft endothelium, by reducing damage in the peri-operative period, rather than inducing host EC-chimerism might be an efficacious strategy to reduce TA development.

Rosiglitazone attenuates TA development, but impairs systemic VSMC function

Immunosuppressive therapy intends to target the recipient's immune system to dampen the alloreactive response and to preserve the transplanted organ, but while it succeeded to prevent the acute rejection episodes, little advances were made in preventing and treating TA. Beside strategies aiming at influencing the immune response, direct modulation of VSMC as well as endothelial and adventitial cell responses to both immunologic and non-immunologic assaults might be considered as an alternative strategy to intervene in the development of TA.[34] Therefore, in **Chapter 4** the efficacy of the thiazolidinedione rosiglitazone (RSG), a synthetic peroxisome proliferator-activated receptor-γ (PPARγ) agonist, to attenuate TA development was studied as well as mechanisms involved. Using the aorta transplant model in rats, RSG was shown to significantly reduce the development of TA. The underlying mechanism appeared to be plural since RSG was shown to 1) reduce alloreactive T cell responses, which was however not mediated through upregulated frequency and function of $CD4^+CD25^+FoxP3^+$ regulatory T cells, and 2) reduce intragraft

expression of SDF-1α and PDGFRβ (a receptor for PDGF). Reduced intragraft expression together with the observed reduced PDGF-induced proliferation of medial and neointimal VSMCs *in vitro* in the presence of RSG favor for a direct effect of RSG on VSMC proliferation. Furthermore, reduced expression levels of SDF-1α, a potent chemoattractant for progenitor cells, within the neointima following *in vivo* treatment with RSG, favors for reduced recruitment and homing of SMPCs into the injured allograft vascular wall as a putative mechanism for reduced TA development.

Endothelial dysfunction is a key player in cardiovascular pathology as endothelium has a crucial role in maintaining normal vessel wall function by its ability to inhibit thrombus formation, leukocyte adhesion, VSMC proliferation and by regulation of vascular tone. Endothelial dysfunction is the triggering event in the proliferative and fibrotic processes involved in atherosclerosis and associated complications and it is an independent predictor of future cardiovascular events.[35,36] In the transplantation setting, beside the vascular tree inside the transplanted organ, the recipient vasculature outside the graft is affected, supposedly due to transplant-associated systemic inflammatory status which will have deleterious effects on systemic endothelial cell function[37,38], and thereby increasing the risk for cardiovascular events after transplantation.[39] Therefore, in **Chapter 5** the effect of *in vivo* RSG treatment on the development of systemic EC dysfunction was studied. Alike clinical solid organ transplantation, experimental allogeneic aorta transplantation resulted in the development of transplantation-induced systemic EC dysfunction. RSG treatment resulted in an overall improvement of EC-dependent relaxation in response to metacholine as measured *ex vivo* using isolated thoracic aorta rings in an organ-bath setup. However, *in vivo* exposure to RSG decreased vasorelaxation indicative of an impaired dilatory response of VSMCs in response to the EC-independent vasodilator sodium nitrite. Although the direct negative effect of RSG on VSMC function was compensated by the general improvement of EC-function and there is an overall RSG-induced improvement of vasodilator function, nonetheless, the long-term effects of RSG treatment on endothelial-independent relaxation and therefore, on the risk for cardiovascular events subsequent to allografting remain to be reported. Clinical evaluations by meta-analysis reported that RSG treatment in patients with type 2 diabetes is associated with increased incidence of myocardial infarction and death from cardiovascular causes.[40,41] A possible explanation for these observations could be based on its effect to increase fluid retention and precipitate heart failure.[40] Our data on RSG-induced VSMC impaired dilatory response might offer a mechanistic explanation on increased risk for cardiovascular events in diabetic patients which, in addition to their changes in the structure of microvessels with hypertrophic remodeling of small vessels[42], present an altered VSMC dilatory response. This might aggravate the abnormal myogenic responsiveness already altered in type 2 diabetic patients[43], thereby contributing to increased wall stress for a given intraluminal pressure, which may further stimulate vascular hypertrophy. Although the aforementioned meta-analysis has its limitations[44], more studies actually suggest harmful effects of RSG usage. Caution is therefore required when prescribing RSG to patients who are already at risk for development of cardiovascular diseases including diabetics and renal transplant recipients. Following the results of the meta-analysis published in 2007, the Food

and Drug Administration (FDA) issued a boxed warning that RSG may increase the risk for myocardial ischemic events, including myocardial infarction.[45] Since then, a consensus regarding the risk posed by RSG has emerged among experts[46-48] and in September 2010 the FDA decided to restrict access to RSG.[49] Nonetheless, our results presented in **Chapter 4** and **Chapter 5** as well as a rapidly increasing number of publications have underscored the importance of PPARγ in cardiovascular diseases.[50] Although contra-intuitive based on clinical data, results from *in vitro* and *in vivo* animal models suggest that PPARγ and its selective agonists have a vascular protective role via their beneficial effects on inflammatory response, neointimal VSMC proliferation, progenitor cell recruitment factors, and endothelial function.[51] However, the controversial clinical outcomes indicate an insufficient knowledge of the cardiovascular biology of the nuclear receptors and the necessity for the development of more effective agonists. Further in-depth studies are necessary to uncover the cardiovascular functions of PPARγ and address its potential and safety use in treatment of occlusive cardiovascular diseases.

Part II: In-Stent Restenosis (ISR)

Neointimal VSMCs and ECs in ISR are non-bone marrow-derived

Another major vascular pathological remodeling process is represented by neointima formation after stent implantation. Development of ISR after endovascular stenting still is a major side-effect resulting in the need for re-revascularization interventions. Although the use of drug-eluting stents have resulted in a decrease in the incidence of ISR[52,53], development of ISR can still not be prevented.[54] The phenotype and the origin of vascular cells as well as the molecular pathways involved in vascular cell recruitment, differentiation and proliferation need to be clarified in order to design novel effective strategies aiming at further attenuating ISR development. Alike TA, it is generally accepted that the main constituents of the neointima in ISR are α-actin-positive VSMCs or myofibroblasts. Their anatomical origin and recruiting mechanisms are still a matter of debate. Recent findings obtained in our lab and by others favor for a role of recirculating vascular progenitor cells, which may be partly derived from the BM as demonstrated in the development of TA and restenosis in experimental models in rodents.[24,25,55-60] The involvement of BM-derived vascular cells in the development of ISR is, however, as yet not clear, although the presence of BM-derived cells in human atherectomy samples has been suggested.[61] However, from this study it remains unclear whether the BM-derived cells detected in the neointima were already present in the atherosclerotic lesion before stenting. Therefore, in **Chapter 6,** the contribution of BM-derived VSMCs in the development of ISR without established atherosclerosis was analyzed using an experimental abdominal aorta stenting model in transgenic (hPAP) BM-chimeric rats. This model allowed identification of BM-derived vascular cells recruited in response to stenting. However, results obtained indicated that virtually all neointimal VSMCs were of non-BM origin and only infiltrating $CD45^+$ leukocytes turned out to be derived from the BM compartment, in line with results in a rat

balloon injury model with a small population of neointimal cells coming from BM-derived progenitors.[62] In wire-induced injury of the femoral artery in mice, Daniel *et al.* recently showed that most of the BM-derived cells found in the NI were monocytes/macrophages, and there was no apparent substantial long-term contribution of these cells to the cellular mass of the NI. Moreover, their results provide evidence that the definite differentiation of BM-progenitor cells into VSMCs or ECs is only an exceedingly rare event.[63] These finding were confirmed by another recent study showing that even though BM-derived α-smooth muscle actin-expressing cells do infiltrate injured vessels in several models of vascular injury, they do not fully diferentiate in VSMCs.[64] These new findings favor for a local origin of VSMCs in NI formation. The precise anatomical origin of neointimal VSMCs in ISR remain to be determined and may include (recirculating) vascular progenitor cells residing the vascular wall.[5,65,66] Vascular wall resident progenitor cells where recently isolated from vasculogenic niches in the media[67] and the adventitia[68-70] of the vascular wall. Although BM-derived cells seem to play a minor role in neointima formation in experimental ISR, this does not exclude the possibility that early after stenting BM-derived cells are recruited to the injured vascular wall and create a microenvironment in which local progenitor cells niches are activated and mobilized by BM-derived cells in a paracrine fashion. Rodriguez-Menocal *et al.* showed in a rat balloon injury model that BM-derived monocytes/macrophages were abundantly present in the media and adventitia of injured vessels in early stages but their number declined in the vascular wall with time.[62]

Long-term experimental Type 1 diabetes enhances ISR

The incidence of macrovascular disease (*i.e.* atherosclerosis), including peripheral and coronary artery disease is increased in diabetic patients in comparison to nondiabetic patients.[71-78] Hyperglycemia can promote vascular complications by multiple mechanisms[79] resulting in diabetes-associated vascular disease, characterized by systemic endothelial cell dysfunction[80] and structural changes of large and small arteries leading to tissue hypoperfusion and hypoxia. Moreover, diabetic patients present worsened clinical outcome and repeated re-vascularizations after percutaneous coronary intervention with stent placement[81] and as yet the precise underlying mechanism is unknown. In order to get more insight into the mechanism underlying enhanced development of ISR in diabetes, reliable long-term diabetic models are necessary. Therefore, in **Chapter 7** a novel rat model for long-term hyperglycemia was established and used to study the development of ISR following stenting in the abdominal aorta using age-matched non-diabetic rats as controls. This model was developed in diabetes prone BioBreeding (BBDP) rats with long-term impaired glycemic control. BBDP rats develop immune-mediated diabetes resembling human Type 1 diabetes since it develops during adolescence and involves a disorder of the immune system.[82] Poorly controlled diabetes in BBDP rats was maintained by suboptimal treatment with insulin pellets resulting in chronically elevated blood glucose and HbA_{1c} levels. After stent implantation, the diabetic rats presented increased neointima formation compared with control rats. This novel experimental model reflecting poorly controlled Type 1 diabetes can therefore be used to study the augmented severity of ISR allowing more

in-depth analyses on the mechanism(s) involved. In addition, this model might constitute a useful tool in investigating the profile of circulating vascular progenitor cells associated with a poor glycemic control and offers the possibility to determine the effect of the diabetic metabolic environment on progenitor cell mobilization after vascular injury. Increased NI hyperplasia in diabetics might be caused by a disequilibrium between various classes of progenitor cells. While SMPCs might contribute to neointima hyperplasia as decreased SMPC number is associated with reduced neointima formation[83,84], EPCs might prevent it, replacing the damaged endothelium.[85] Increased SMPCs is the sign of a deleterious profibrotic status resulting in adverse remodeling of damaged tissue in diabetes. In a mouse model of type 1 diabetes increased number of SMPCs with the involvement of TGF-β/BMP-6 axis as an important modulator for these cells was described.[86] Moreover, Nguyen *et al.* showed that blood from diabetic patients yielded higher numbers of myofibroblast progenitor cells than blood from control subjects, presenting increased proliferation and decreased apoptosis.[87]

Long-term experimental Type 1 diabetes impairs angiogenic potential of the vascular wall

The long-term diabetic model described above was then used to investigate the presence of progenitor cells in the vascular wall and the effect of the hyperglycemic status on their function. As shown in Chapter 6, BM-derived cells had none or a limited contribution to ISR. Possible mechanisms by which progenitor cells may contribute to intimal hyperplasia would be to directly adhere to the vascular wall and building up neointima "inside-in" or entering vascular wall via the perivascular *vasa vasorum*, migrate to the neointima from the "outside-in." Therefore, in **Chapter 8**, the presence of angiogenic cells residing in the vessel wall was investigated and the long-term effect of diabetes on their function was analyzed. Using *ex vivo* cultured aorta rings obtained from long-term hyperglycemic rats as well as from their age-matched controls, the angiogenic potential of the cells residing in the vessel wall was assessed. We showed that sprouting in long-term diabetic rats was significantly impaired compared with non-diabetic age-matched control animals. These results from isolated vessels show that the effects on neovascularization include deficient angiogenic sprouting of vascular wall resident cells, independent of effects of recirculating cells or their secreted pro- or anti-angiogenic factors. We already showed that non-BM-derived progenitor cells contribute to neointima formation in ISR, and at least some part of them might originate from the vasculogenic zone of vascular wall itself. The vascular wall was described to harbor both smooth muscle and endothelial progenitor cells.[67-69,88] An impaired ability of vascular wall resident EPCs to migrate and proliferate, along with the already proven deficiency in number and function of circulating EPCs in diabetic condition[89-92] would lead to impaired reendothelialization, favoring the neointima formation. In addition to direct participation to reendothelialization, these local angiogenic cells might have a paracrine function by promoting the homing and proliferation of circulating EPCs. The deficient angiogenic potential of diabetic vascular wall cells would also impair the generation of neo-vessels by angiogenesis, thereby worsening the recovery from an ischemic event. Local delivery of agents that could influence the number and the quality of vascular wall resident

EPCs, along with a systemic double edged therapy in order to increase circulating EPC and reduce SMPC frequency, would be a possible approach to reduce neointima formation.

TA and ISR: a matter of a disturbed EPC and SMPC balance?

Mobilization and recruitment of circulating and/or tissue-resident progenitor cells that can give rise to ECs and VSMCs play an important role in neointima formation. Maintaining the fine balance between the "beneficial" EPCs and "deleterious" SMPCs in favor of proliferation, migration and homing of the EPCs and diminishing the number of SMPCs would be an efficient approach in decreasing the rate and the extent of vascular remodeling in both TA and ISR. In end-stage renal disease patients who have an increased incidence of atherosclerotic cardiovascular disease, a decreased number of EPCs was found, whereas the number of SMPCs remained unaffected, suggesting that an imbalance between the two population could negatively affect vascular remodeling.[93] Nonetheless, caution should be taken when considering progenitor cell manipulation, as there is no definitive "good" or "bad" vascular progenitor cell as both endothelial and smooth muscle progenitors may act as a double-edged sword in the pathogenesis of arteriosclerosis.[88] Moreover, both EPCs and SMPCs may even derive from a common precursor and EPCs can transdiferentiate in SMPCs which may complicate cell-based therapies.[94,95] Ideally one drug would control the microenvironment in which progenitor cells reside and/or are recruited in such a way that their subsequent differentiation into either ECs or VSMCs would eventually lead to reduced neointima formation. Moreover, such a drug would promote both effects by simultaneously targeting two different signal pathways within same tissue microenvironment resulting in opposite and biologically complementary effects. Several drugs have beneficial effects on EPCs like PPARγ agonists[96] and statins[97], and in the same time inhibit VSMC proliferation and therefore reduce neointimal formation.

Concluding perspectives

Figure 1 summarizes several contributors to the pathogenetic mechanism(s) involved in TA and ISR development as revealed in the experimental work discussed in this thesis. They all represent possible interventional areas in controlling neointima hyperplasia.

Neointima formation is a complex pathological process with both humoral and cellular participants, influenced by local and systemic factors. Injury of endothelium is the initiating event in both TA and ISR development and the severity of EC damage correlated with the magnitude of TA formation. Preventing endothelial damage and preserving its function in the first place (improved transplantation and stenting procedures) would be the ideal approach in preventing NI development. Nonetheless, with all caution taken, EC damage appears and the need to reduce activation (and further damage) following transplantation as well as to enhance reendothelialization of mechanically-injured vessels is warranted. Both circulating and vascular resident wall EPC might contribute in different phases and

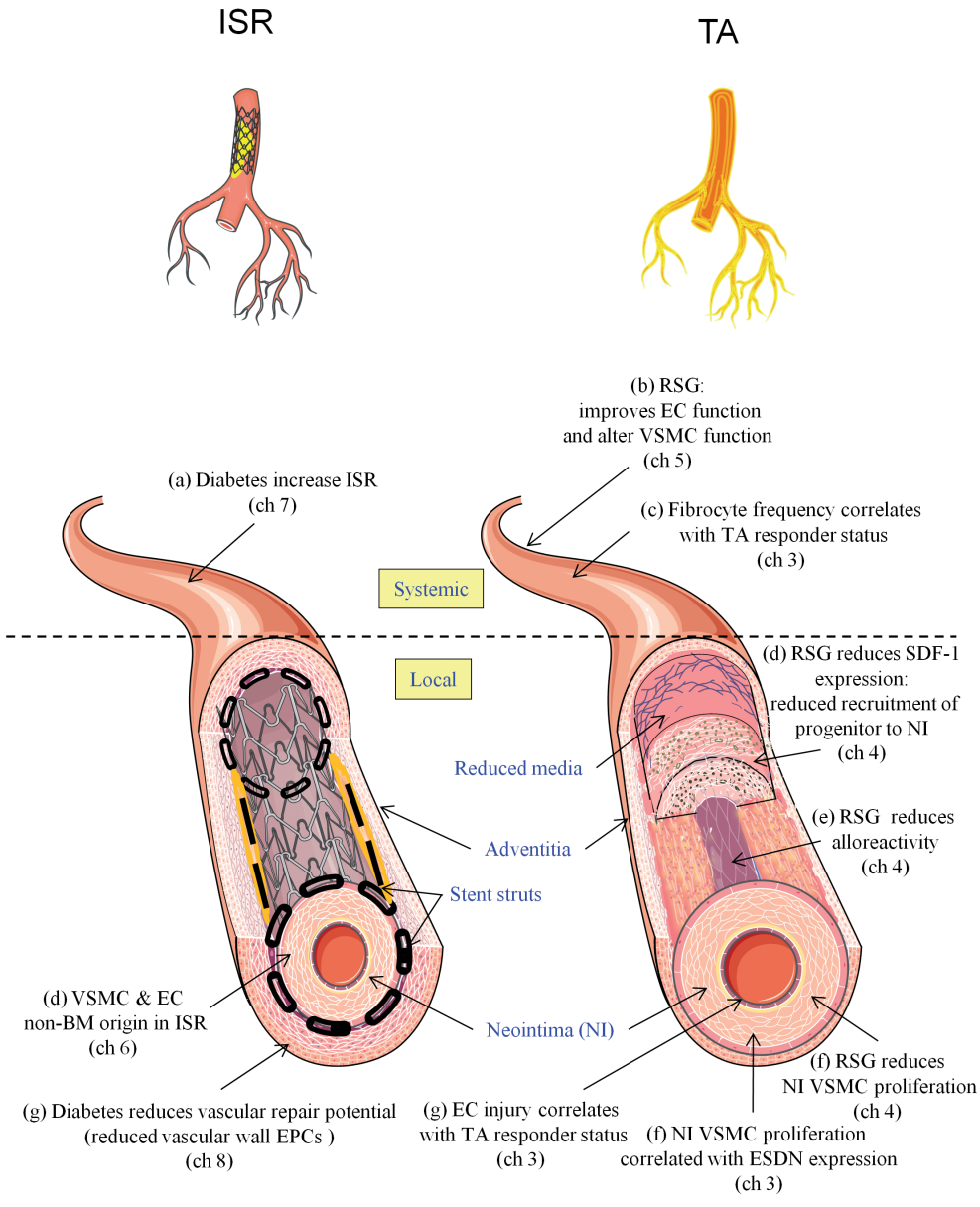

Figure 1. Possible interventional targets to prevent and treat neointima hyperplasia addressed in this thesis. In-stent restenosis (ISR, top left): neointima formation after revascularization by stent placement in an atherosclerotic plaque with a focal distribution in the vascular tree. Transplant arteriosclerosis (TA, top right): characteristic concentric intimal thickening with a diffuse distribution of neointima hyperplasia throughout the vascular tree. Systemic interventional strategies: (a) control risk factors (diabetes), (b) improve systemic EC function, (c) diminish the pro-fibrotic status (decreasing circulating smooth muscle progenitors). Local interventional strategies: (d) modulate progenitor cell recruitment (decreased SMPCs and increased EPCs), (e) reduce inflammation (reducing endothelial and medial damage), (f) reduce VSMC proliferation, (g) maintain endothelium integrity or favor reendothelialization (enhance local angiogenic potential). Figure was produced using Servier Medical Art (www.servier.com).

proportion to recover the endothelial lining. Therefore, potentially efficacious therapies might include modulation of vascular progenitor cell subsets to facilitate vascular repair by increasing EPC number and homing capacity to the site of injury. Also, controlling debilitating factors like diabetes (PPARγ agonists) will improve the attempts to maintain a healthy endothelium.

VSMCs are the major players in NI formation and therefore their recruitment, migration and proliferation form all potential targets for therapeutic interventions. Some of currently used immunosuppressive drugs like rapamycin, mycophenolic acid, cyclosporin, calcium channel blockers, and statins are known to possess anti-proliferative properties. As inhibiting VSMC proliferation does not succeed to prevent NI, therapies aimed at earlier stages (*i.e.* before their homing and proliferation is mounted) might be a more efficient approach. Targeting SMPCs recruitment and homing (*e.g.* by SDF-1/CXCR4 blockade) in conjunction with local control of proliferative factors (like PPARγ or ESDN), may offer a more efficient preventive and therapeutic strategy for controlling NI development.

Although much progress was made in the last decade regarding vascular progenitor cell biology and their different sources, their efficient use in therapeutic schemes is just at early stages. To specifically target the vascular progenitor cell and modulate their behavior in order to limit NI, a more complex understanding of the multitude of factors regulating their biology is required. There are still a lot of unanswered remaining issues/questions like: 1. It is not yet fully established what the frequency and the degree of contribution of these cells is in NI formation, 2. What is the percentage of cells that are derived from different sources, 3. What factors do actually determine their recruitment, 4. What is the role of mature endothelial and smooth muscle cell interaction with progenitor cells, 5. What are the mechanisms that influence the recruitment of either a common progenitor cell for ECs and VSMCs and/or of separate progenitors for the different cells lineages, and 6. What molecular mechanisms determine the direction of their differentiation.

Both TA and ISR represent major macrovascular complications in treating end-stage organ failure and occlusive atherosclerotic diseases, respectively. Both diseases are characterized by EC damage, inflammation and NI hyperplasia. The additional cardiovascular risk factors, like diabetes, influencing these impaired responses to vascular injury, require multifactorial and multi-level complex therapies. At present there are no efficacious therapeutic measure to control their onset and evolution. The lack of adequate prevention and therapeutic protocols impels for further research aiming at unraveling their pathogenetic mechanisms. Knowledge on mechanisms will enable the development of more efficacious interventional strategies specific for various stages of the diseases meant for inhibiting or limiting their evolution. Knowing how they arise, is knowing how to control them.

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10

Nederlandse Samenvatting voor niet-ingewijden

Transplantaat Arteriosclerosis en In-Stent Restenosis

Experimentele studies over pathomechanismen en therapeutische interventie

Sumarul în limba română

Arterioscleroza Transplantului şi Restenoza în Stent

Studii experimentale despre mecanismele patologice şi intervenţii terapeutice

Nederlandse Samenvatting voor niet-ingewijden

Doel van het onderzoek was het bestuderen van verschillende cellulaire en/of moleculaire processen die ten grondslag liggen aan het vernauwen van bloedvaten in getransplanteerde organen (transplantaat arteriosclerosis [TA]) en na stenting (in-stent restenosis [ISR]). TA en ISR vertegenwoordigen twee belangrijke bloedvat-vernauwende afwijkingen aan slagaders, die beide gekenmerkt worden door endotheelschade, ontsteking en vorming van neointima (NI).

Bloedvaten zijn samengesteld uit drie concentrische lagen, namelijk de intima met endotheelcellen (ECs) die in direct contact komen met het circulerende bloed, de media met hierin gladde spiercellen (VSMCs) en de adventitia. De neointima (NI) is een "abnormale laag" die ontstaat binnen het bloedvat en die de normale bloedcirculatie zal verminderen of stoppen. De belangrijkste cellen in de neointima zijn VSMCs met hiertussen enkele ontstekingscellen en aan de lumenzijde afgedekt met ECs. Als gevolg van de vaatvernauwingen komen er onvoldoende zuurstof en voedingsstoffen in de achter de vernauwing gelegen organen wat leidt tot ischemie. Deze ischemie zal uiteindelijk resulteren in weefselschade en dus achteruitgang in het functioneren van deze organen. TA bestaat uit NI-vorming in bloedvaten van getransplanteerde organen als gevolg van de afstotingsreactie van het afweersysteem van de ontvanger, die gericht is tegen het 'lichaamsvreemde' getransplanteerde orgaan. ISR bestaat uit NI-vorming in bloedvaten na stenten, waardoor vernauwing van het vaatlumen in deze bloedvaten optreedt. Een stent is een metalen buisje dat wordt ingebracht in een bloedvat met een vernauwing als gevolg van atherosclerose, met het doel de bloedstroom in het bloedvat te herstellen. Hoewel de stent het bloedvat zal heropenen, zal het probleem daardoor slechts tijdelijk worden verholpen, doordat na een bepaalde periode zich aan de binnenkant van de stent opnieuw neointima zal vormen, die leidt tot ISR.

Er zijn geen adequate therapieën voorhanden waarmee TA en ISR kunnen worden voorkomen of behandeld. In een poging om betere strategieën te ontwikkelen om deze macrovasculaire complicaties te voorkomen en te behandelen, is het noodzakelijk om beter te begrijpen in welke vormen zij voorkomen en hoe zij zich ontwikkelen. Het doel van de studies die in dit proefschrift worden gepresenteerd, was om meer inzicht te krijgen in de cellulaire en moleculaire mechanismen die ten grondslag liggen aan de ontwikkeling van TA en ISR. De aandacht ging vooral uit naar het verkrijgen van nieuwe inzichten in het herstel van beschadigd endotheel, naar de herkomst van VSMCs in de NI en naar de factoren die verantwoordelijk zijn voor VSMC-rekrutering en VSMC-celdeling in TA en ISR. Het onderzoek werd uitgevoerd in zowel pre-klinische *in vivo*-modellen als *in vitro*-modellen. De belangrijkste uitkomsten van dit onderzoek luiden als volgt.

TA is op de lange termijn de belangrijkste oorzaak van transplantaatverlies en sterfte bij ontvangers van harttransplantaten. In de klinische situatie is er variatie tussen transplantaatontvangers in zowel de mate van afstoting als in het lange termijn resultaat. Deze variatie suggereert een genetische gevoeligheid voor TA-ontwikkeling, die tot op heden nog niet volledig kan worden verklaard. In **Hoofdstuk 3** hebben we laten zien, dat

net als tussen mensen ook tussen verschillende rattenstammen verschillen in de mate van TA-ontwikkeling optreden. Om dit te onderzoeken hebben we een studie gedaan bij ratten waarin een stukje aorta is getransplanteerd. In deze aorta ontwikkelt zich namelijk ook een neointima en deze neointima is daarom een goed model voor wat er in de vaten van de getransplanteerde organen plaatsvindt. Ongecontroleerde celdeling van spiercellen in de neointima wordt gezien als de centrale gebeurtenis gedurende de ontwikkeling van TA, die uiteindelijk resulteert in volledige afsluiting van de bloedvaten in het transplantaat. Het verschil in snelheid waarmee TA tot ontwikkeling komt, was gecorreleerd met de proliferatieve capaciteit van VSMCs in de neointima en een toename van het aantal VSMC-voorlopercellen in het bloed. Behoud van een intact en gezond endotheel remt NI-vorming. Transplantatieprocedures en afstotingsepisodes beschadigen de endotheliale laag, die vervolgens moet worden gerepareerd met andere ECs. We toonden aan dat ratten met een verhoogde TA ook een verhoogde vervanging van ECs kenden. De toegenomen noodzaak om het endotheel te herstellen kan de weerspiegeling zijn van een eerdere en meer agressieve, acute vasculaire afstoting en daarom ook van een ernstiger beschadiging van de bloedvaten. Daarom zou het behoud van transplantaat-endotheel een mogelijke manier zijn om TA te verminderen.

Aangezien er geen adequate therapie beschikbaar is om TA af te remmen, is in **Hoofdstuk 4** het effect van rosiglitazone op TA-ontwikkeling geanalyseerd. Rosiglitazone, een zogenaamde PPARγ-agonist, wordt al lange tijd gebruikt bij type 2 diabetes patiënten voor een betere bloedsuikerbalans, maar het effect op TA-ontwikkeling was niet bekend. Bij ratten werd de abdominale aorta getransplanteerd en daarna werden deze behandeld met rosiglitazone. Ratten die behandeld werden met rosiglitazone kenden minder NI-vorming dan niet-behandelde ratten. Rosiglitazone had een meervoudig gunstig effect, door vermindering van de afstotingsreactie, door vermindering van neointimale VSMC-proliferatie en door signaalfactoren die neointimale voorlopercellen recruteren.

Omdat ontstekingen (afstotingsreacties) bij transplantaties worden geassocieerd met systemische endotheeldisfunctie (endotheeldisfunctie buiten het transplantaat), hebben wij in **Hoofdstuk 5** bestudeerd of rosiglitazone ook een gunstig effect laat zien op systemische endotheelfunctie. Dit bleek inderdaad het geval te zijn. Echter, in de afgelopen jaren zijn er cardiovasculaire bijwerkingen van dit medicijn beschreven bij gebruik door diabetespatiënten. Zo is er een vergroot risico op een hartinfarct. In ons model laten wij ook zien dat rosiglitazone een negatief effect had op de VSMC-functie, maar dit effect werd gecompenseerd door een verbeterde endotheelfunctie. Verder onderzoek is nog nodig om volledig te begrijpen hoe dit medicijn werkt en ingrijpt op de vaatfunctie.

ISR is de meest voorkomende, levensbedreigende, complicatie na het aanbrengen van een stent. De anatomische bron van neointimale VSMCs in ISR is nog niet bekend. In **Hoofdstuk 6** onderzochten we daarom de oorsprong van neointimale VSMCs in experimentele ISR. Stents werden geïmplanteerd in de abdominale aorta van de ratten en na een maand vormde zich een NI binnenin de stent. We hebben de herkomst van NI-cellen geanalyseerd en toonden aan dat de meeste van deze cellen niet afkomstig waren uit het beenmerg.

Diabetes (suikerziekte) is een bekende cardiovasculaire risicofactor. Diabetespatiënten hebben een verhoogde kans op ISR. Diabetische diermodellen zijn nuttig voor het onderzoeken van de mechanismen van ISR in de diabetische populatie. Een dergelijk diermodel was echter nog niet beschreven. We ontwikkelden daarom een nieuw diabetisch ratmodel voor de lange termijn met langdurig hoge bloedsuiker waarden, zoals beschreven in **Hoofdstuk 7**. Met behulp van stent implantatie in de aorta hebben we laten zien dat ISR in ons model toeneemt door diabetes. De diabetische ratten werden daarnaast gekenmerkt door verhoogde eiwit concentraties in de urine, veel urine productie en hoge bloedsuikerwaarden. Deze resultaten valideren dit nieuwe type 1-diabetesmodel voor het bestuderen van de mechanismen van ISR onder diabetische condities.

Omdat de cellen die NI vormen, niet afkomstig zijn uit het beenmerg, zou één van de mogelijke oorsprongen de vaatwand zelf kunnen zijn. Daarom onderzochten wij in **Hoofdstuk 8** het effect van diabetes op de functie van de voorlopercellen die in de vaatwand zèlf voorkomen. We gebruikten aorta's uit ons nieuwe type 1-diabetesmodel en uit niet-diabetische controle ratten. We toonden aan dat de uitgroei van kleine bloedvaatjes uit de vaatwand bij de lange termijn diabetische ratten significant verminderde in vergelijking met niet-diabetische, leeftijd-gematchte controle dieren. Hiervoor werd een *in vitro* model gebruikt, waarbij ringetjes van de aorta van ratten in een groeifactorrijke gel geplaatst worden. We gebruikten aorta's van ons nieuwe type 1-diabetesmodel (zoals beschreven in Hoofdstuk 7) en van niet-diabetische controle ratten. Vanuit de aorta ringetjes groeien nieuwe vaatjes, waarbij verondersteld wordt dat dat gebeurt vanuit een gespecialiseerd soort lokale vaatwandvoorlopercellen, die lijken op endotheelvoorlopercellen. De nieuwgevormde vaatjes van diabetische ratten waren korter en geïsoleerde cellen uit deze nieuwe vaatjes hadden een vertraagde celdeling. Deze resultaten tonen aan dat bij diabetes niet alleen de circulerende endotheelvoorlopercellen, maar ook voorlopercellen voor nieuwe vaten in de vaatwand zèlf gestoord zijn in hun functie. Diabetes kan dus ook het cardiovasculaire risico verhogen via een direct effect op de potentiële voorlopercellen die bijdragen aan NI-ontwikkeling.

TA en ISR zijn beide oorzaken van macrovasculaire complicaties bij de behandeling van de eindstadia van respectievelijk orgaanfalen en occlusieve atherosclerotische ziekten. Beide ziekten worden gekenmerkt door EC-schade, ontsteking en NI-vorming. Momenteel zijn er nog geen afdoende therapeutische methoden om hun ontstaan en ontwikkeling te controleren. Verder onderzoek is noodzakelijk om hun mechanismen te ontdekken. Onze resultaten hebben het inzicht in het ontstaan van TA en ISR vergroot en kunnen bijdragen aan de ontwikkeling van nieuwe methoden om TA en ISR te voorkomen of te behandelen. Weten hoe ze ontstaan, is weten hoe ze te bestrijden!

Sumarul în limba română

Arterioscleroza Transplantului şi Restenoza în Stent

Studii experimentale despre mecanismele patologice şi intervenţii terapeutice

Arterioscleroza transplantului (AT) şi restenoza în stent (RIS) sunt două boli vasculare caracterizate de remodelare vasculară obliterantă. Remodelarea vasculară este un termen larg folosit ce descrie alterări structurale ale peretelui vascular, rezultând în îngroşarea acestuia cu pierderea elasticităţii şi a funcţiei normale. Vasele de sânge sunt alcătuite din trei straturi celulare concentrice: tunica internă sau endoteliu cu celulele endoteliale, tunica medie, un ţesut muscular neted ce conţine celule musculare netede, şi tunica externă sau adventiţia. Neointima (NI) este un strat celular anormal ce se formează în interiorul vaselor de sânge, la nivelul intimei şi mediei, lucru care conduce la scăderea sau chiar oprirea circulaţiei normale a sângelui. Ca urmare, organele vor fi insuficient vascularizate, ducând la ischemia şi disfuncţia lor. Arterioscleroza transplantului e caracterizată de formarea neointimei în vasele organelor transplantate datorită rejetului cronic. Restenoza în stent e caracterizată de formarea neointimei în interiorul stentului. Stentul este un cilindru metalic care se introduce în vasele obturate de ateroscleroză pentru a redeschide lumenul şi a restabili circulaţia sangvină. Implantarea stenturilor este acum o metodă de tratament recunoscută şi larg răspândită în terapia intervenţională în caz de obstrucţie a vaselor care vascularizează cordul. În cazul în care unul (sau mai multe) vase nu furnizează suficient sânge ariei corespunzătoare, există risc de infarct cardiac, o complicaţie a aterosclerozei care poate ameninţa viaţa. Totuşi, chiar dacă stentul implantat va recanaliza vasul, acesta reprezintă doar o soluţie temporară a problemei pentru că, după o perioadă, neointima se va forma pe pereţii interiori ai stentului, conducând la restenoza în stent. În prezent, nu există o terapie eficientă pentru prevenţia şi tratamentul AT şi RIS. De aceea, în încercarea de a dezvolta strategii terapeutice mai eficiente, se depun eforturi pentru a înţelege mai bine modalitatea de apariţie şi evoluţie a acestor boli macrovasculare. Scopul studiilor prezentate în această teză este de a aduce noi informaţii în ce priveşte mecanismele care stau la baza apariţiei AT şi RIS, care pot deveni potenţiale zone de intervenţie terapeutică.

Neointima e compusă din celule musculare netede, matrix extracelular şi celule inflamatorii. Acestea formează un strat celular fibros pe partea luminală (internă) a vaselor de sânge. În prezent, atât originea anatomică exactă a celulelor care formează NI, cât şi factorii care contribuie la selectarea şi aglomerarea lor nu sunt complet cunoscuţi. De aceea, folosind modele experimentale *in-vitro* şi *in-vivo*, o atenţie aparte în această teză a fost acordată rolului jucat de celule vasculare progenitoare ale celulelor ce formează neointima, cât şi potenţialului lor terapeutic. În prima parte a tezei (capitolele 3-5) au fost cercetate mecanismele care duc la apariţia arteriosclerozei transplantului, folosind modelul de transplant aortic pe şobolani. Au fost investigate rolul celulelor musculare netede şi al celulelor lor progenitoare în formarea NI, rolul reconstrucţiei endoteliului în procesul de dezvoltare a arteriosclerozei de transplant, precum şi rolul tratamentului cu rosiglitazonă în reducerea NI. Partea a doua a tezei (capitolele 6-8) a investigat diferite aspecte ale

mecanismelor de apariție a restenozei în stent folosind modelul de stentare a aortei pe șobolani. Au fost investigate atât contribuția celulelor provenind din circulație versus cea a celulelor provenind din măduva osoasă, cât și influența diabetului în formarea neointimei.

Capitolul 1 oferă o privire generală asupra diferitelor aspecte care caracterizează procesul de remodelare vasculară care stă la baza formării AT și RIS, cu descrierea diferențelor și similarităților dintre mecanismele lor patogenice. O atenție specială a fost acordată rolului central pe care tunica internă (endoteliul) îl are în faza inițială de dezvoltare a AT și RIS, capacității de proliferare a celulelor musculare netede și potențialei contribuții a celulelor vasculare progenitoare în formarea neointimei. **Capitolul 2** introduce pe scurt studiile experimentale descrise în capitolele care-l urmează.

Arterioscleroza transplantului e principala cauză de degenerare a transplantului și de mortalitate, pe termen lung, în cazul transplantului cardiac, fiind semnul definitoriu de rejet cronic. În cazul pacienților transplantați, primind același regim imunosupresor și având aceeași incompatibilitate cu grefa, s-a constatat că există variație în ce privește rata rejetului și rezultatul pe termen lung. Această diferență între pacienți sugerează o predispoziție genetică pentru AT, care nu a fost încă pe deplin explicată. În **Capitolul 3** am studiat rata de apariție și dezvoltare a AT în transplantul de aortă pe șobolani, folosind diferite combinații de transplant între diferite linii genetice, și am analizat posibilele mecanisme celulare și moleculare care ar putea sta la baza ratei de dezvoltare diferite a AT. Folosind șobolani Lewis (Lew) și Brown Norway (BN) ca donori și primitori în diferite combinații de transplant, a fost analizată apariția și evoluția AT în timp. Ca și în cazurile clinice, am constatat diferențe în ce privește rata de apariție și dezvoltare a AT între diferite tipuri de șobolani. În contrast cu șobolanii primitori de tip Lew, care au dezvoltat AT gradual, șobolanii primitori de tip BN au dezvoltat maxim de AT în termen de 4 săptămâni de la transplant, față de 24 de săptămâni în cazul Lew. Severitatea AT în ambele tipuri de primitor a fost în final aceeași ca magnitudine a neointimei, ceea ce a variat a fost rata lor de apariție și evoluție. Apariția mai rapidă a AT în primitorii de tip BN a fost corelată atât cu potențialul proliferant intrinsec crescut al celulelor musculare netede din neointimă, cât și cu expresia crescută a unei gene, ESDN, care a fost descrisă ca un indicator al formării neointimei. În plus, dezvoltarea mai rapidă a AT s-a corelat cu frecvența crescută a fibrocitelor în circulația sangvină, acestea fiind considerate a fi potențiale celule progenitoare ale celulelor musculare netede din neointima. Prezența unui endoteliu intact și funcțional inhibă formarea neointimei. Procedurile de transplant și episoadele de rejet distrug stratul de celule endotelial iar acesta are nevoie să fie refăcut de către alte celule endoteliale. Aceste celule pot proveni din grefa însăși sau din organismul primitor. În combinațiile de transplant folosite, am arătat că șobolanii de tip BN (cei care au dezvoltat AT mai rapid) au o rată mai mare de reconstrucție cu celule provenind din organismul primitor (de tip BN) decât în cazul șobolanilor de tip Lew, c-o rată de înlocuire a celulelor endoteliale mai mică. Această rată crescută de reconstrucție a endoteliului în cazul șobolanilor ce dezvoltă mai rapid AT, sugerează o necesitate crescută de refacere a endoteliului și, deci, o distrugerea endotelială mai rapidă și mai masivă, semn al unei reject mai sever. Aceste rezultate indică faptul că, mai degrabă, mijloacele de prezervare intactă

a endoteliului în timpul transplantului, decât promovarea refacerii acestuia, ar putea da rezultate mai bune în ce priveşte reducerea dezvoltării arteriosclerozei transplantului.

Deoarece nu există încă o terapie eficientă pentru a împiedica sau reduce AT, în **Capitolul 4** a fost investigat efectul pe care tratamentul cu rosiglitazonă îl are asupra AT. Rosiglitazona e un compus folosit în clinică pentru a creşte senzitivitatea la insulină în cazul pacienţilor cu diabet de tip 2, însă efectele sale în cazul transplantului nu sunt cunoscute. În acest studiu am arătat că neointima la şobolanii care au primit transplant de aortă şi au fost trataţi cu rosiglitazonă a fost semnificativ redusă faţă de cei transplantaţi dar netrataţi cu acest medicament. Acest rezultat benefic s-a datorat, cel puţin în parte, unui efect inhibitor asupra proliferării celulelor musculare netede din neointimă, reducerii procesului imun de rejet şi prin efecte asupra factorilor care contribuie la atragerea celulelor vasculare progenitoare în neointima.

Deoarece statusul inflamator cronic asociat transplantului este asociat cu o disfuncţie endotelială sistemică (disfuncţie a endoteliului din afara organului transplantat), în **Capitolul 5**, am arătat că tratamentul cu rosiglitazonă are, deasemenea, un efect benefic şi asupra funcţiei sistemice endoteliale. Totuşi, în pofida tuturor aceste efecte pozitive dovedite în studiile experimentale, în ultimii ani au apărut tot mai multe consemnări ale efectelor secundare cardiovasculare negative la pacienţii trataţi cu rosiglitazonă (o creştere a riscului de infarct miocardic). De aceea, în prezent, prescrierea de rosiglitazonă se face cu foarte mare precauţie. În acest capitol, pe lângă efectul benefic asupra funcţiei endoteliale, am arătat că într-adevăr rosiglitazona afectează negativ funcţia celulelor musculare netede din tunica medie, lucru care ar putea, în parte, sta la baza efectelor negative vasculare. În continuare, rolul acestui medicament (şi al celor din aceeaşi clasă cu el) în patologia cardiovasculară este încă neclar şi studii viitoare sunt necesare pentru a înţelege modul lor de acţiune.

Restenoza în stent este cea mai comună complicaţie actuală în terapia vasculară intervenţională cu implantarea de stent, punând în pericol viaţa pacientului. Originea anatomică precisă a celulelor musculare netede care formează neointima nu este precis determinată. Se vehiculează mai multe ipoteze asupra originii lor, cum ar fi cea medulară (celulele vasculare progenitoare care intră în alcătuirea neointimei provenind din măduva osoasă), circulatorie (celule vasculare progenitoare provenind din circulaţia sangvină), organică (provenienţa din diferite organe: ficat, inimă, ţesut adipos) sau celule progenitoare care provin din peretele vascular însuşi. În **Capitolul 6** am studiat originea celulelor care formează neointima în modelul de stentare aortică pe şobolani şi am arătat că cele mai multe dintre ele nu proveneau din măduva osoasă.

Diabetul zaharat reprezintă un factor major de risc cardiovascular. Pentru a studia mecanismele implicate este nevoie de folosirea unor modele experimentale care să se apropie cât mai mult de situaţia clinică. În **Capitolul 7** este prezentat un nou model experimental pe şobolani pentru diabetul de tip 1, care prezintă mai multe avantaje: diabetul are etiologie autoimună (şi nu indus de substanţe toxice care pot interfera cu mecanismele patologice de studiat), poate fi menţinut şi urmărit pe o perioadă îndelungată de timp (7-8 luni de zile, echivalentul a aprox. jumătate din durata de viaţă a unui şobolan), costuri mai mici decât în

cazul folosirii animalelor experimentale mai mari (cum ar fi porcul). Şobolanii cu diabet au prezentat hiperglicemie, poliurie, proteinurie. Pentru validare, am stentat un grup de animale diabetice şi le-am comparat cu un grup control, fără diabet, de aceeaşi vârstă. În modelul experimental de diabet cu durată îndelungată restenoza în stent a fost accelerată. Rezultatele obținute validează acest nou model experimental ca unul util şi fiabil pentru studierea mecanismelor prin care diabetul zaharat contribuie la accelerarea restenozei în stent.

Deoarece celulele care formează neointima, în cazul restenozei în stent, nu provin din măduvă, aşa precum am arătat în capitolul 6, un posibil loc de origine al lor ar putea fi reprezentat de peretele vascular însuşi, care s-a raportat că deține celule progenitoare vasculare. În **Capitolul 8** am investigat efectul diabetului zaharat asupra celulelor prezente în peretele vascular şi care au potențial de a forma noi vase de sânge (potențial angiogenic). În acest studiu am folosit o cultură *ex-vivo* de inele de aortă. Pentru aceasta, au fost izolate aorte provenind de la modelul dezvoltat în capitolul 7 (şobolani cu diabet pe termen lung) şi au fost tăiate în inele de aproximativ 1 mm, care au fost apoi cultivate într-un mediu cu factori de creştere specifici pentru celulele endoteliale. În acelaşi timp, inele aortice obținute de la şobolani fără diabet au fost cultivate pentru a servi drept control. Din aorta şobolanilor diabetici au crescut vase de sânge mai puține şi mai scurte decât din cea a şobolanilor normali. Majoritatea celulelor din noile vase de sânge crescute din inelele de aortă au fost identificate ca celule endoteliale. Acest lucru indică faptul că diabetul zaharat afectează celule progenitoare din peretele vascular (celule progenitoare endoteliale), alterând potențialul lor angiogenic. Diabetul zaharat poate, astfel, să crească riscul cardiovascular (de exemplu, de restenoză în stent printr-o refacere întârziată a endoteliului) şi prin efectul direct pe care-l are asupra celulelor progenitoare din peretele vaselor de sânge.

În **Capitolul 9** datele prezentate în capitolele anterioare sunt discutate şi puse într-o perspectivă mai largă.

Atât AT cât şi RIS reprezintă două complicații macrovasculare majore în tratamentul stadiului final de cedare a organelor şi, respectiv, a aterosclerozei ocluzive. Ambele sunt caracterizate de distrugere endotelială, inflamație şi formarea neointimei. Nu există în prezent o terapie eficace de a controla apariția şi evoluția lor şi de aceea studii de cercetare viitoare sunt necesare pentru a descoperi, mai departe, mecanismele lor patogenice. Ştiind cum apar, vom şti şi cum să le controlăm.

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